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# Interaction of Penicillin with the Bacterial Cell: Penicillin-Binding Proteins and Penicillin-Sensitive Enzymes

PETER M. BLUMBERG[1] AND JACK L. STROMINGER

*The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138*



## INTRODUCTION

### General

The beta-lactam antibiotics stand out among antimicrobial agents for their wide spectrum of activity and for their remarkably low toxicity to animals. These substances are composed of two classes, the penicillins and cephalosporins (see Fig. 1). The central feature of the antibiotic nucleus is the highly strained beta-lactam ring. As discussed below, this ring may be that part of the molecule which reacts with the penicillin target. The side chains attached to the nucleus are of importance because of their ease of chemical modification and the profound effects which they exert on the properties of the molecule (162). Different side chains can, for exam-

[1] Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

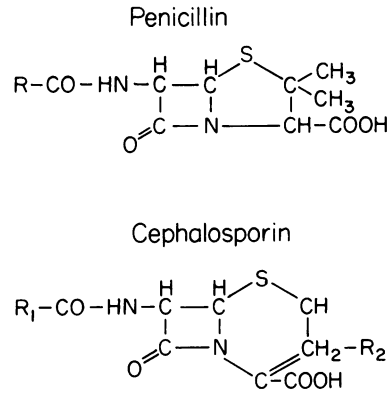



FIG. 1. *Structures of penicillin and cephalosporin.*

ple, render the penicillin resistant to degradation by penicillinase, tolerant of gastric acidity, or able to penetrate the outer envelope of gram-negative organisms.

Since the introduction of penicillin into general medical practice, a continual problem has been the emergence of drug-resistant strains of bacteria (201). In many cases this resistance has been shown to arise from production of beta-lactamase (penicillinase), an enzyme which degrades the antibiotic. In other cases, the intrinsic sensitivity of the organism is what appears to have been altered. Synthesis of novel penicillin derivatives has fortunately kept pace so far with the appearance of penicillin-resistant bacterial strains. In order to maintain such progress, a detailed understanding of the mode of action of penicillin in killing bacteria, and the nature of the interaction between penicillin and its receptor should be valuable.

The remarkably low toxicity of penicillin for animals suggests that the drug inhibits some bacterial structure or function without analogue in higher organisms. In fact, the target of penicillin has been demonstrated to be the bacterial cell wall, a unique microbial structure. (A trivial exception to this generalization exists [204]. The cephalosporin 7-(5-benzyl-thioacetamido)-cephem-3-ylmethyl-*N*-dimethyldithiocarbamate-4-carboxylic acid also inhibits protein synthesis and thus is active against both fungi and bacterial spheroplasts. Apparently, the cephalosporin decomposes to liberate the dimethyldithiocarbamate in the 3-position on the molecule. This hydrolysis product is responsible for the unusual antibiotic behavior.) The cell wall is a giant macromolecule which envelopes the organism, supporting the bacterial cell membrane against lysis caused by the difference in osmolarity between the cell cytoplasm and the culture medium, which is relatively hypotonic. Because cell wall synthesized in the presence of penicillin is weak and unable to provide the cell with this needed osmotic support, penicillin lyses growing bacteria. Consistent with this explanation, penicillin does not kill cells growing in sufficiently hypertonic medium. Likewise, bacteria are protected from the action of penicillin in the absence of cell growth, in which case new cell wall synthesis is not required.

The action of penicillin as an inhibitor of cell wall biosynthesis offers a second important reason for studying the interaction of this drug with the bacterial cell. Many fundamental, but poorly understood, phenomena are related to cell wall synthesis: morphogenesis—the cell wall is responsible for maintaining the shape of the bacterium. What regulates the shape assumed by the cell wall? Cell division—division is by definition the formation of a septum containing cell wall and cell membrane to yield two daughter cells. Membrane proteins—many of the proteins involved in cell wall synthesis are membrane bound, including most of the penicillin receptors. Regulation of complex synthetic pathways—cell wall synthesis involves the interplay of large numbers of enzymes and is closely related as well to the synthesis of the other macromolecules in the cell. Penicillin provides a valuable tool for investigation of some of these problems.

## Cell Wall Structure and Synthesis

The bacterial cell wall is a highly complex structure consisting of multiple classes of polymers. These polymers include the peptidoglycan, teichoic acids, attached and secreted polysaccharides or proteins, or both, and, in gram-negative organisms, lipopolysaccharide. The state of knowledge regarding these structures has been ably discussed in recent reviews, which cover cell walls in general (94, 174, 199, 208, 227), peptidoglycan (83, 86, 93, 210, 217, 226, 244), teichoic acids (4, 7, 8, 9), and lipopolysaccharide (129, 136, 165). Among these structures, the peptidoglycan is of particular importance. Its integrity is required for maintenance of cell shape in bacteria. Moreover, the peptidoglycan is the one cell wall constituent universally distributed in bacteria (except in extreme halophiles and in mycoplasma, both of which exist in very specialized environments), and, unlike most of the other cell wall polymers, it is essential for cell survival under normal growth conditions.

The precise composition of the peptidoglycan varies with the bacterial species. Typical struc-

tures are those shown in Fig. 2. The peptidoglycan, also known as murein or mucopeptide, possesses a heteroglycan backbone of alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid. The *N*-acetyl-muramic acid residues are substituted by peptide chains which are cross-linked to give a mesh-like character to the peptidoglycan. The net result is to knit the entire bacterial cell wall into a "bag-shaped macromolecule" (236).

Synthesis of peptidoglycan can be divided conceptually into three stages (217, 218, 220). The first step, catalyzed on the inside of the cell by cytoplasmic enzymes, is the synthesis of the soluble peptidoglycan precursor uridine 5'-diphosphate (UDP)-*N*-acetylmuramyl-pentapeptide (Fig. 3). This step is followed by transfer of the *N*-acetylmuramyl-pentapeptide and *N*-acetylglucosamine to a lipid carrier, $C_{55}$-isoprenyl alcohol, in the membranes, forming a subunit of the glycan polymer (Fig. 4). The membrane-bound disaccharide pentapeptide is now modified in a species-specific fashion. Such modification may include substitution of a carboxyl group on glutamic or diaminopimelic acid residues or attachment of a peptide side chain (e.g., the pentaglycine chain attached to the lysine residue of the pentapeptide in *Staphylococcus aureus* [Fig. 4]).

In the last stage, the modified disaccharide-pentapeptide residue is transferred to a glycan acceptor on the outside of the cell to form linear peptidoglycan. Finally, the peptide chains of the linear peptidoglycan are cross-linked in a reaction catalyzed by a transpeptidase. It is this last step which is the penicillin-sensitive reaction in cell wall synthesis. The cross-bridge is formed between the carboxyl group of the penultimate D-alanine in the pentapeptide on one chain and an amino group in a nearby chain, for example, that of the diaminopimelic acid residue (Fig. 5a). When a peptide cross-bridge is present, e.g., the pentaglycine chain in *S. aureus*, the amino group of the cross-bridge is used instead (Fig. 5b). The driving force for the cross-linking is provided by release of the terminal D-alanine from the pentapeptide chain and probably by the insolubility of the cross-linked product. By using this transpeptidation mechanism, the organism avoids the energetic problem that would be involved in achieving net synthesis of peptide bonds outside the cell.

In some organisms, a D-alanine carboxypeptidase activity is present in addition to the transpeptidase. This activity, found in *Escherichia coli* and *Bacillus subtilis* but not in *S. aureus*, cleaves the terminal D-alanine from the pentapeptide chain of several different substrates. Its function is presently only conjectural. Because the tetrapeptide resulting from the action of the carboxypeptidase cannot function as a donor in transpeptidation, one hypothesis is that its function may be to decrease the amount of cross-linking in the cell wall of organisms possessing this enzyme.

## Identification of the Target of Penicillin Action

The effect of penicillin on cell wall structure and morphology has been known since the 1940s (59, 84, 85). Duguid's early analysis of the morphological effects showed great perspicacity (59). He observed not only that penicillin required cell growth for activity, but also that cell death appeared to be caused by cell lysis: "The

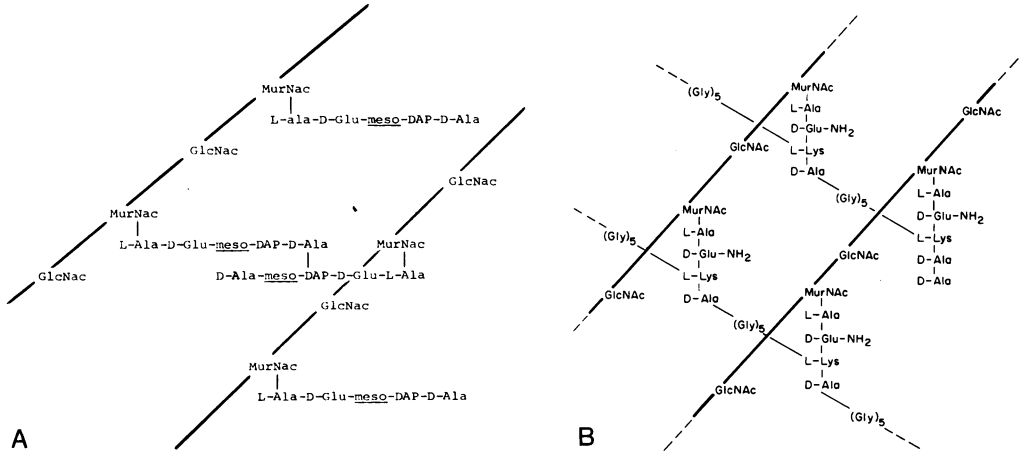



FIG. 2. *Structure of the peptidoglycan. A, Peptidoglycan of Escherichia coli. B, Peptidoglycan of Staphylococcus aureus. MurNac, N-acetylmuramic acid; GlcNac, N-acetylglucosamine.*

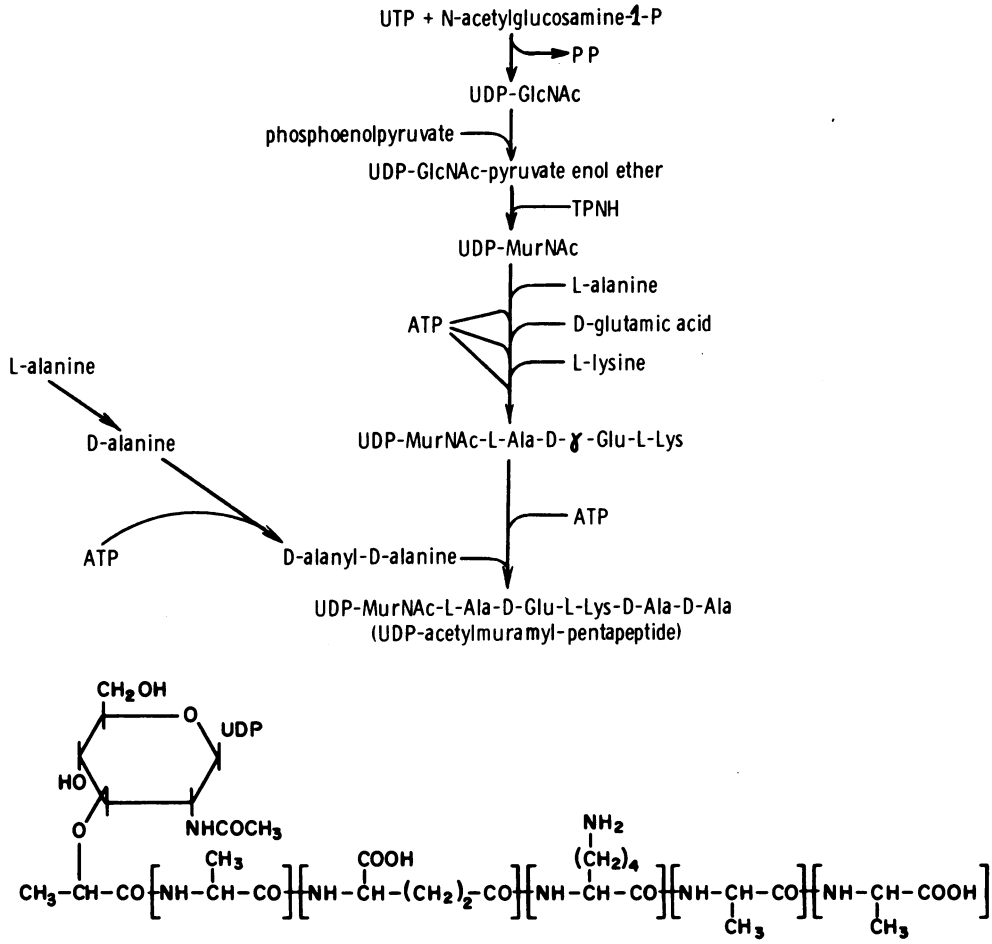



FIG. 3. *First stage in cell wall synthesis: formation of UDP-N-acetylmuramyl-pentapeptide (structure shown).*

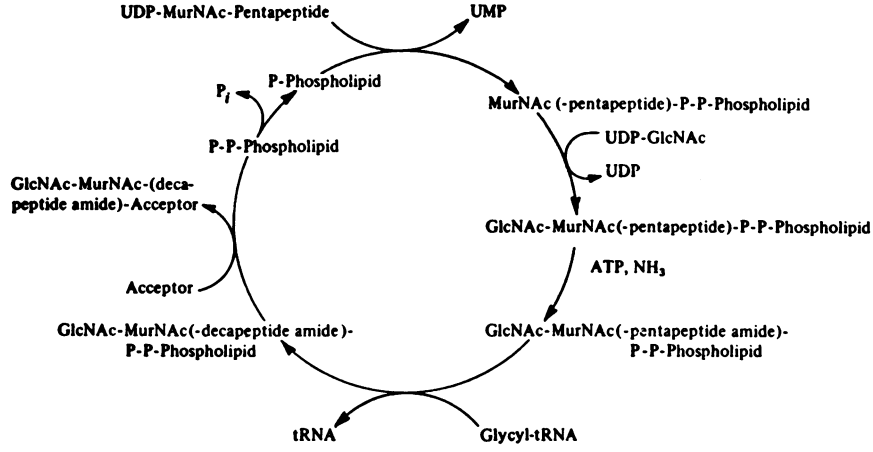



FIG. 4. *Second stage in cell wall synthesis. The modification reactions illustrated are those which occur in Staphylococcus aureus. The lipid moiety is a* $[C_{55}]$*isoprenyl alcohol* $[H(CH_2—\overset{CH_3}{\overset{|}{C}}=CH—CH_2)_{11}OH]$.

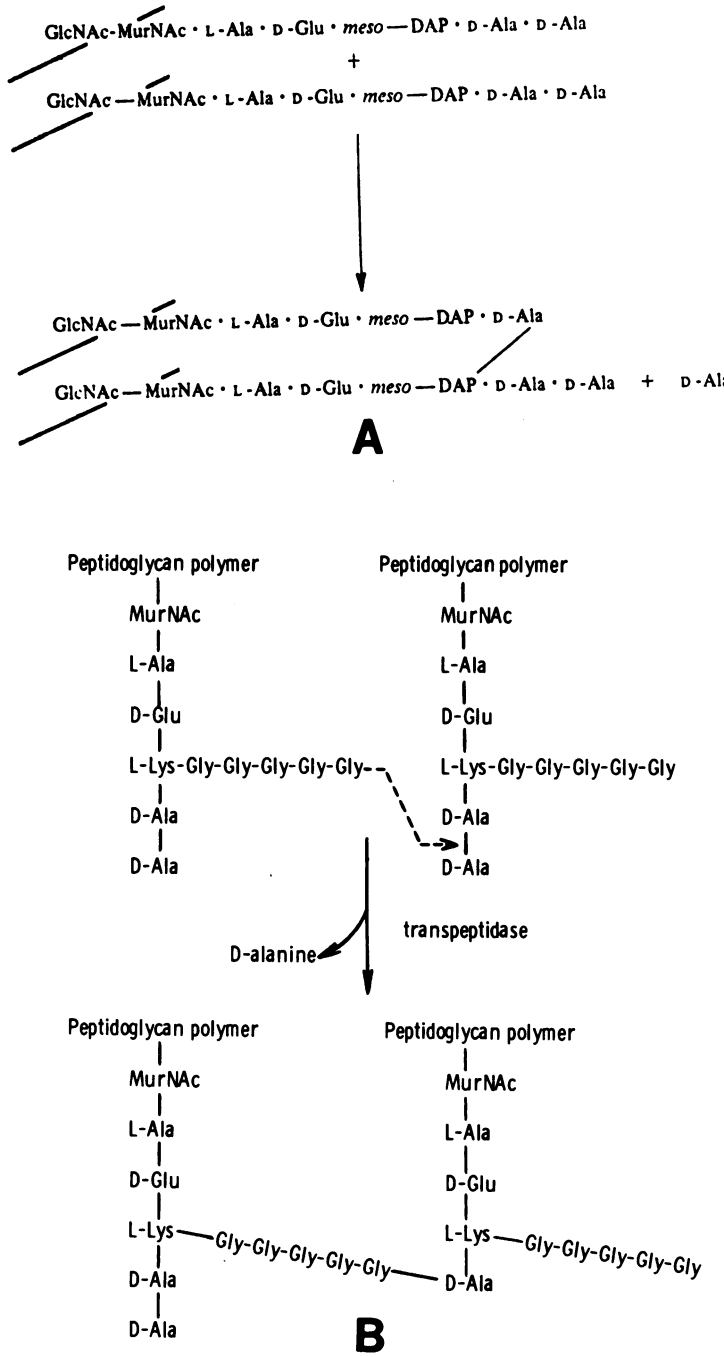



FIG. 5. *Final stage in cell wall synthesis: cross-linking of peptidoglycan polymers by transpeptidation. A, Escherichia coli; B, Staphylococcus aureus.*

morphological changes produced by the lower penicillin concentrations . . . suggest that penicillin at these concentrations interferes specifically with the formation of the outer supporting cell wall, while otherwise allowing growth to proceed until the organism finally bursts its defective envelope and so undergoes lysis." A decade later, these observations had been con-

firmed and extended by the finding that such penicillin-treated cells could be protected from the action of the antibiotic by the presence of an osmotic support, either sucrose or sodium chloride (97, 116, 117). This result demonstrated a causal link between the observed changes in cell morphology, lysis, and cell death.

Elucidation of the specific lesion in cell wall synthesis caused by penicillin followed from a different line of investigation. Early important observations were that labile esters, apparently containing reducing sugars (181), and acid soluble absorbing materials, presumably free nucleotides (155), accumulated in cells inhibited by penicillin. Determination of the structure of these nucleotide-sugar compounds (175–177, 216) revealed that they included UDP-*N*-acetylmuramyl-pentapeptide. This finding suggested that penicillin blocked a stage in cell wall biosynthesis subsequent to synthesis of this nucleotide precursor (182, 221). These stages are now known to include both glycan polymerization and peptide cross-linking. Martin and co-workers (137–140) in studying the composition of the peptidoglycan of the cell wall of *Proteus mirabilis* were led at first to the hypothesis that penicillin was inhibiting cross-linking, though later analyses led them to doubt their earlier conclusion (109). Wise and Park (243) and Tipper and Strominger (228, 229) on the basis of different studies of the effects of penicillin on peptidoglycan synthesis in *S. aureus* concluded that the terminal reaction was a transpeptidation and that penicillin specifically blocked this reaction. (However, the generality of this conclusion has not been established).

This conclusion that penicillin blocked cross-linking was supported by in vitro studies. Transfer of sugar-peptide from nucleotide precursor to lipid and acceptor was not inhibited by large excesses of penicillin (33, 142, 143, 147); the sole remaining reaction which penicillin might inhibit in the biosynthetic sequence was thus transpeptidation. Finally, development of an in vitro transpeptidation assay permitted the direct demonstration of penicillin action (2, 105, 106).

The model of transpeptidation and the proposed mode of action of penicillin which evolved from the above studies were as follows (222, 228, 229): the transpeptidase was hypothesized to react with the peptide bond between the terminal D-alanines in the pentapeptide chain of uncross-linked peptidoglycan. An acyl-enzyme intermediate would be formed and D-alanine released. The amino group from the prospective cross-bridge would next displace the enzyme from the acyl-enzyme intermediate, regenerating free enzyme and forming a peptide cross-bridge (Fig. 6).

Penicillin was hypothesized to be an analogue of the terminal D-alanyl-D-alanine in the pentapeptide chain (Fig. 7). The CO-N bond in the highly strained beta-lactam ring would correspond to the peptide bond cleaved during transpeptidation, and penicillin might, in fact, be an analogue of the transition state in peptide bond cleavage. The transpeptidase would react with penicillin to split the beta-lactam ring to form a penicilloyl-enzyme complex, analogous to the postulated acyl-enzyme intermediate. However, since the penicilloyl-enzyme complex is stable, the transpeptidase would be inactivated (Fig. 6).

The above brief description of cell wall synthesis does not emphasize the complexity of the phenomenon. Regulation of wall synthesis is tied into deoxyribonucleic acid (DNA) replication and the cell cycle. A specific cell shape is maintained during growth. The amount of wall synthesis is varied with the growth rate. The levels of degradative and synthetic enzymes are preserved in careful balance.

Recently, evidence of this complexity has begun to appear from several laboratories (219). Multiple penicillin targets exist. How many are there? What are their relationships to each other and their roles in cell wall metabolism? Furthermore, the precise manner in which penicillin inhibits its various targets has never been fully elucidated. Is penicillin actually a substrate analogue or might it act at an allosteric site, as recently suggested (123)? Does penicillin always act in the same way? The discussion which follows will examine the evidence regarding these questions.

## PENICILLIN-BINDING COMPONENTS

Penicillin was first demonstrated to bind to bacteria in the late 1940s (50, 51, 134, 135, 203). Its interaction with the cell has subsequently been studied in considerable detail (1, 6, 44–46, 49–51, 55, 58, 63–65, 67, 69, 75, 76, 110, 111, 113–115, 134, 135, 157, 160, 179, 185, 188, 189, 192, 197, 200, 202, 203, 209, 211, 225). An early discussion of the subject which showed great insight, but which is now out of date, is that of Cooper (48). The goal of the penicillin-binding studies has been twofold, to elucidate the interaction of penicillin with the drug receptor(s) and to investigate the roles these receptors play in the metabolism of the cell. The state of understanding of both questions is still incomplete. However, the application of modern biochemical techniques has led to dramatic progress over the last few years. It has been possible

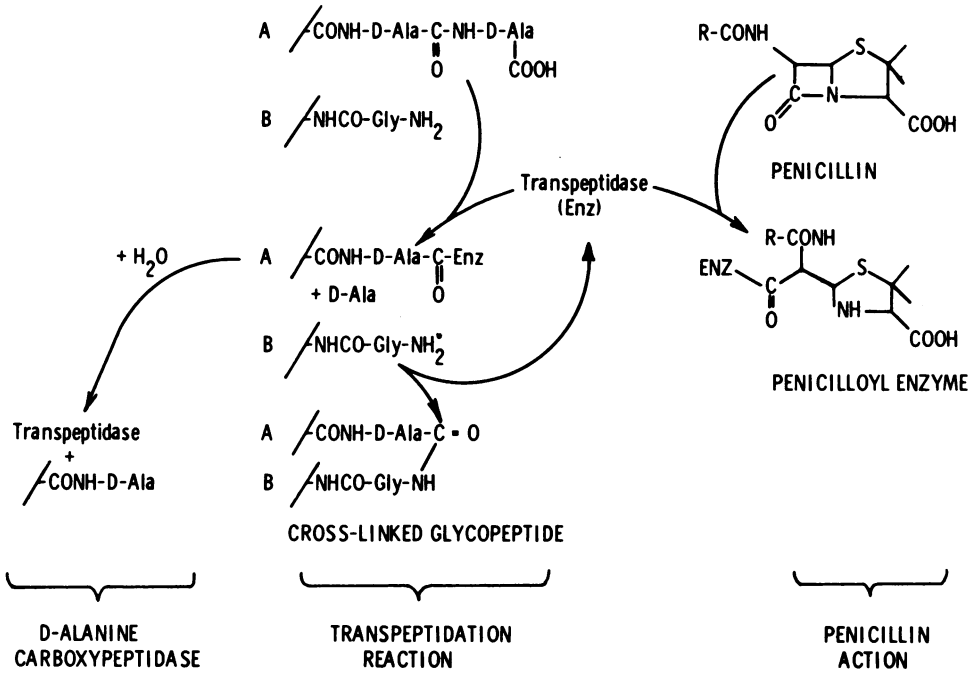



FIG. 6. *Proposed mechanism of transpeptidation and its relationship to penicilloylation and* D*-alanine carboxypeptidase activity. A represents the end of the main peptide chain of the glycan strand. B represents the end of the pentaglycine substituent from an adjacent strand. If the acyl enzyme intermediate can react with water instead of the acceptor (left), the enzyme would be regenerated and the substrate released. The overall reaction would be the hydrolysis of the terminal* D*-alanine residue of the substrate (*D*-alanine carboxypeptidase activity).*

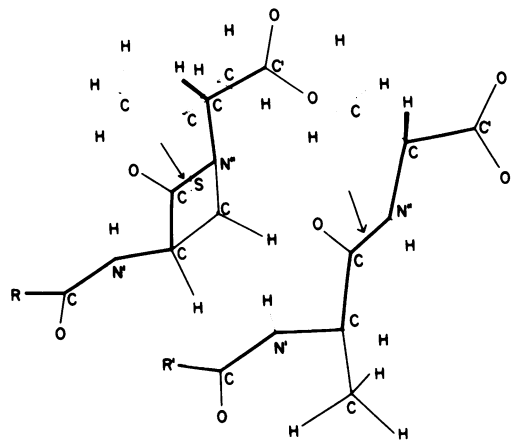


FIG. 7. *Dreiding stereomodels of penicillin (left) and of the* D*-alanyl-*D*-alanine end of the peptidoglycan strand (right). Arrows indicate the position of the CO-N bond in the beta-lactam ring of penicillin and of the CO-N bond in* D*-alanyl-*D*-alanine at the end of the peptidoglycan strand (228).*

to demonstrate multiple targets for penicillin. The roles of some of these targets have been identified. Furthermore, the penicillin-binding components have been isolated either as a mixture, or, for one of the components, in pure form. By using this material, the interaction of penicillin with its receptors has been shown to be much more complicated than previously believed.

The amount of penicillin which bacteria bind is very small. As a result, early efforts to detect the removal of penicillin from culture by bacteria were unsuccessful. Specific binding was obscured by the background loss of penicillin due to decomposition. Demonstration of binding therefore had to await the synthesis of radioactive penicillin, labeled in one of two ways. Early studies often used $^{35}S$-labeled penicillin produced biosynthetically by growth of the penicillin-producing mold in the presence of radioactive sulfate. The chief advantage of this material was its high specific activity, up to 300 mCi/mmol (209). This permitted studies which would not have been possible with material of lower specific activity (209). Its major drawback was the relatively short half-life of $^{35}S$, only 90 days. The alternative strategy was to use penicillin radioactively labeled with $^{14}C$ in the side chain attached to the penicillin nucleus. This product, unlike the $^{35}S$-labeled material, is now available commercially. Unfortunately, its spe-

cific activity has generally been in the range of 25 mCi/mmol (the theoretical maximal activity for substitution by a single atom of $^{14}C$ is 60 mCi/matom), which has been inconveniently low for many studies of penicillin binding.

### What Is Actually Bound?

In general, the entire penicillin molecule appears to react with its target, and a covalent bond appears to be formed between the carbonyl group of the beta-lactam ring and an unidentified group on the penicillin-binding components (PBCs) (see later section). In addition to this specific binding, certain penicillin degradation products may also bind to cells. Although no good comparisons have been published, cells appear to bind comparable quantities of penicillin regardless of whether the site of labeling of the antibiotic is in the side chain or in the drug nucleus (64, 114). Further evidence that the entire molecule is bound comes from studies of the products liberated upon release of the penicillin bound to the binding components. The $^{35}S$-labeled compound released from the PBCs of *M. pyogenes* by alkaline treatment co-chromatographed with penicilloic acid (209). Moreover, derivatives and degradation products of this material behaved chromatographically in the same way as did the comparable products arising from penicilloic acid. More recent studies with the membrane-bound PBCs of *B. subtilis* showed that the product of reversal by hydroxylamine co-chromatographed with authentic penicilloyl hydroxymate in four solvent systems (114). After reversal by ethanethiol and methylation with diazomethane, one of the two products co-chromatographed with authentic $\alpha$-ethylthio-$\beta$-methylpenicilloate (114). The other had slightly slower mobility in all four solvent systems examined. The significance of this latter observation is not yet known.

The above conclusion that the entire penicillin molecule is bound ought not to be generalized to the case of cephalosporins, because it is known that hydrolysis of the beta-lactam bond of the cephalosporin leads to expulsion of the side chain in the 3-position (171). A comparable reaction may occur after cephalosporin binding to PBC, but this matter has not been investigated.

An early question was whether the binding of radioactive penicillin which was observed was a property of the penicillin per se or whether it was due to an impurity in the radioactive material. The above work strongly implied that penicillin itself bound to the PBC (the penicilloic acid released by alkali from *M. pyogenes* presumably was a degradation product of the bound material, because penicilloic acid itself does not bind [49]). Further evidence was that uptake of the radioactive material was prevented by prior addition of pure, unlabeled penicillin (45, 50, 157, 202).

A characteristic of this specific uptake of radioactive penicillin was that after the PBCs became saturated, subsequent addition of radioactive penicillin had no effect on the amount of binding. However, in addition to this specific binding, a second binding phase was also sometimes observed, which was proportional to the amount of penicillin added and which could not readily be saturated. This second phase, unlike the specific uptake, was insensitive to such treatments as boiling of the PBCs. As shown by Cooper et al. and others (46, 49, 197), such nonspecific binding was due to degradation products in the penicillin which accumulated with time; the nonspecific binding could be eliminated by repurification of the radioactive penicillin immediately before use. Although the identity of the penicillin degradation products responsible for such nonspecific binding has not been completely established, the most likely candidate is penicillenic acid. Unlike the other degradation products which Daniel and Johnson examined, penicillenic acid can compete for binding with radioactive penicillin (55). Furthermore, it can also be shown to bind nonspecifically to proteins (55, 119).

### How Much Penicillin Do Cells Bind?

Determinations of the amount of penicillin bound by bacteria vary with the growth state of the organisms, their treatment, etc. As a general rule, gram-positive organisms bind 4 to 15 nmol of penicillin per g (dry weight) (48, 225). The gram-negative organism *E. coli* may bind 5- to 10-fold less. Such numbers correspond to 100 to 10,000 molecules of penicillin per cell (47, 64, 67, 134, 191, 225); the range of values commonly reported is 1,000 to 4,000 (64).

The major uncertainty in these values lies in determinations of the number of cells per g (dry weight). For example, Suginaka et al. (225) and Rogers (197) both reported *S. aureus* H to bind specifically 5.7 nmol of penicillin per g (dry weight). However, the number of molecules per cell were reported as 4,000 and 100, respectively. In the first case, the number of cells was determined from a viable count. As recognized by the authors, this value may be low because clumps and chains of organisms are counted as a single organism. Rogers, in contrast, counted

total cells. This latter method includes non-viable particles and, in addition, tends to give artifactually high values (149).

A second cause for uncertainty, in the case of organisms which bind penicillin only at higher concentrations and in small amounts, is that the background arising from nonspecific binding may be substantial. *E. coli*, which binds one-tenth as much penicillin as does *B. subtilis*, is a typical example (225). Here, 50% of the binding is nonspecific, and special care must be taken to determine the appropriate blank.

### Is the Penicillin Bound Covalently?

No general study has been made of bacterial proteins which bind penicillin tightly, but in a readily reversible fashion; the only work along such lines has been the analysis of cell wall enzymes which have later turned out to be reversibly inhibited by penicillins (see section on penicillin-sensitive enzymes). Instead, effort has concentrated on that firm penicillin binding which requires relatively drastic treatment for reversal. The evidence indicates that this binding is covalent. The nature of the covalent bond, however, is not certain.

Bound radioactive penicillin cannot be eluted from either whole cells or membranes by washing with nonradioactive penicillin (44, 64, 114, 134, 202) or released by treatment with penicillinase (114). Conceivably the binding could be noncovalent, but very tight. However, protein-denaturing agents also do not induce penicillin release. Thus, treatment with sodium dodecyl sulfate (15, 16, 209) at 100 C, boiling (16, 46), exposure to 8 M urea (16, 225) or 6 M guanidine-hydrochloride (16) or phenol extraction (44, 202) have no effect. Evidence that such procedures actually do denature the PBCs is the ability of these treatments to prevent subsequent specific binding of radioactive penicillin. Similarly, Pronase digestion of the purified D-alanine carboxypeptidase-penicillin complex from *Bacillus stearothermophilus* did not release intact penicillin (16). Further evidence for a covalent linkage of penicillin to the PBCs is formation of an $\alpha$-ethylthio-penicilloate upon treatment of the PBC-bound penicillin with ethanethiol under conditions where the ethanethiol would not have been predicted to react with intact penicillin (114).

### To What Group Does Penicillin Bind?

Early workers (209) believed that the penicillin might be bound as an ester because of its alkali lability. More recently the bond between the penicillin and the PBCs has been suggested to be a thioester, at least in the case of the *B. subtilis* binding components. The major evidence for this conclusion is the characteristics of reversal of the penicillin binding by various agents. In particular, hydroxylamine and $H_2O_2$ release penicillin under conditions where normal amide and ester linkages allegedly are not cleaved (114, 225). Furthermore, treatment with ethanethiol is reported to lead to penicillin release (114, 225). Consistent with this explanation, prior reaction of the PBCs with thiol reagents prevents the subsequent binding of radioactive penicillin (114). In addition, with the purified D-alanine carboxypeptidase of *B. subtilis*, which accounts for 70% of the total penicillin binding in that organism (15), binding of penicillin blocks one of the four sulfhydryl groups titratable with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (233). Likewise, thiol reagents inactivate the enzyme (232).

Although the above evidence is suggestive, it is by no means persuasive. The concentrations of thiol reagents employed to block binding by penicillin are grossly excessive. Whereas 1 mM iodoacetamide suffices to block normal sulfhydryl groups, Lawrence et al. (114), for example, used 200 times more to prevent penicillin binding. At such concentrations, the reagent is no longer specific. Although the reason for the high levels of reagent which were necessary may be that the sulfhydryl group is deeply buried on the enzyme so that it is relatively inaccessible to the reagent, alternatively it may be that the alkylating reagent is reacting with a less readily alkylated residue than cysteine. Moreover, the ability of penicillin to protect a sulfhydryl group in the native enzyme from DTNB implies only that the group is in the penicillin binding site, not that it is covalently bound to penicillin. Furthermore, the hydroxylamine-induced release may be an enzymatically catalyzed reaction rather than a chemical hydrolysis (16). Hydroxylamine release of the bound penicillin from *B. subtilis* membranes was prevented by boiling of the membranes after binding of the penicillin (114). An initial interpretation was that the denaturing treatment rendered the penicillin-enzyme bond inaccessible to the hydroxylamine. More recent studies exclude this explanation. If denaturation is effected by agents which unfold proteins, e.g., sodium dodecyl sulfate, hydroxylamine still fails to bring about release (16). Apparently, undenatured binding components are required. The release of bound penicillin by ethanethiol may likewise require active binding components, because its

action also is blocked by prior boiling of the membranes after binding of radioactive penicillin (114). Tentatively, then, the conclusion is that a sulfhydryl group may be present in the penicillin binding site of the *B. subtilis* carboxypeptidase. This group may or may not be involved in covalent binding. Although a number of the experiments discussed above could be repeated and extended, the availability of purified enzymes and PBCs should permit a more definitive approach, viz, the isolation and characterization of peptides containing bound penicillin.

## Under What Conditions Can Penicillin Be Released from the PBCs?

Specific release of penicillin from PBCs occurs upon treatment with $H_2O_2$, ethanethiol, and hydroxylamine as discussed above. The hydroxylamine treatment is of particular note because it does not destroy the ability of the PBCs to combine specifically with penicillin (14). Similarly, in the case of the *B. subtilis* carboxypeptidase, hydroxylamine restored activity to the penicillin-inactivated enzyme (115). This finding was crucial for the later development of covalent affinity chromatography as a means of purifying PBCs (described below) (14).

Even more mild conditions may lead to release of penicillin and regeneration of enzymatic activities. During storage at 6 C in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, in the presence of penicillinase, enzymatic activity slowly returned to penicillin-inactivated D-alanine carboxypeptidase from *B. subtilis*. The rate was dramatically dependent on the penicillin derivative employed. Negligible activity returned when penicillin G or cephalothin was used. In contrast, the half-time for hydrolysis with cloxacillin was 68 h and with 6-aminopenicillanic acid was 14 h (13).

This phenomenon has subsequently been investigated in more detail (16). The D-alanine carboxypeptidases from *B. subtilis* and *Bacillus stearothermophilus* both release their covalently bound penicillin. The rate of release varies with the organism, the specific penicillin derivative, and the temperature. At 37 C, the half-time of release of penicillin G is 200 min for the *B. subtilis* enzyme and 50 to 60 min for the *B. stearothermophilus* enzyme. The rate is highly temperature dependent. At 55 C, the half-time for release of penicillin G from the *B. stearothermophilus* enzyme is only 10 min. This release has the same pH optimum as the enzymatically catalyzed carboxypeptidase reaction.

The nature of the penicillin released by the enzyme is not known. It is neither benzylpenicillin, benzylpenicilloic acid, benzylpenilloic acid (the decarboxylation product of penicilloic acid), benzylpenicillenic acid (a rearrangement product of benzylpenicillin implicated in nonspecific binding), benzylpenillic acid (an acid-catalyzed rearrangement product of benzylpenicillin), nor phenylacetic acid (the penicillin side chain).

As was the case with the hydroxylamine release, the spontaneous release of penicillin would seem to be enzymatically catalyzed; release is prevented by denaturation of the enzyme. An important unresolved question is whether the enzyme simply labilizes the penicillin-enzyme bond, or whether it plays a more direct role, e.g., by transferring penicillin to acceptors such as hydroxylamine or ethanethiol. The determination of the structure of the material released from the enzyme hopefully may distinguish these alternatives.

Penicillinases long have been hypothesized to have evolved from PBCs (190, 228). The demonstration that PBCs can degrade penicillin, albeit at a rate $10^3$ to $10^5$ times more slowly than the usual beta-lactamases (16, 35), would be consistent with this hypothesis.

Multiple proteins are responsible for penicillin binding in most organisms (see later section). The lability of the penicillin-protein bond varies with the particular binding component. Of the five components in *B. subtilis* membranes, component I is the most susceptible to spontaneous cleavage (15). With hydroxylamine-induced cleavage, component I reacts most readily, component V more slowly, and components II and IV more slowly still. Detailed comparisons of rates have not, however, been made (14).

## What Is the Physical Nature of the PBCs?

The penicillin-binding components are proteins found either entirely or predominantly in the cell membrane. The binding components have been isolated from *B. subtilis*, *S. aureus*, and *B. stearothermophilus* by covalent affinity chromatography (14, 245). In the case of the *B. subtilis* carboxypeptidase, only one of two purified PBCs studied in any detail, no lipid or carbohydrate covalently bound to the protein could be demonstrated (232). The amino acid composition of the *B. subtilis* carboxypeptidase was relatively typical (232).

The molecular weights of PBCs have been

determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The values range from a molecular weight of 50,000 for carboxypeptidases from bacilli to approximately 120,000 (15, 245). Such values are relevant to speculation on the origin of penicillinases, because the molecular weights of penicillinases with one exception—the R46 penicillinase—have been reported to be below about 30,000 (35, 36, 196). Therefore, if the two classes of proteins are related, extensive deletion in the PBC gene must be postulated.

Much evidence points to the PBCs being located in the membrane. Vigorous disruption of cells led to liberation of the PBCs into the cell supernatant (51). However, if the cells were broken more gently, most of the PBCs remained in the bacterial cell wall fraction instead (75). The difference in appearance of cell wall fractions in the two preparations suggested that, in the latter case, retention of the cytoplasmic membrane in the wall fraction was responsible for preservation of its penicillin-binding ability. This indeed appears to be the explanation. Pure walls do not bind penicillin (58). Moreover, Cooper (45) showed that the distribution of PBC upon fractionation of cell lysates correlated with distribution of lipid phosphate. Furthermore, the specific binding activity of the lipid fraction was 7 to 12 times that of the whole organisms. Such a value is consistent with the PBCs all being located in the membrane. A difficulty in excluding the presence of a small fraction of soluble PBC was that a certain percentage of the membranes was not recovered during centrifugation. These contaminate the supernatant fraction and may account for the small amount of specific penicillin binding which Cooper reported finding in that fraction.

Similar conclusions were reached by Suginaka et al. (225). Within an experimental error of 20%, the same amount of penicillin was found regardless of whether penicillin binding to whole cells was measured, whether penicillin was first bound to the cells and membranes were subsequently prepared from them, or whether the membranes were prepared first and penicillin was then bound. As before, the qualification on these results is that the 20% experimental error could obscure binding by a biologically significant PBC in the cytoplasm. There is, however, no evidence for such an enzyme.

Little is known about the orientation of the PBCs in the membrane, viz, whether they are all on the outer surface or partly located on both surfaces. At least 50% of the D-alanine carboxypeptidase, the major binding component of *B. subtilis*, is on the outer surface (215). This conclusion is based on the ability of penicillin covalently coupled to Sepharose to inhibit the enzyme in protoplasts. Because steric interference might be expected to prevent complete inhibition, the location of the other 50% cannot be inferred by this method.

### Are PBCs Located in Peripheral Membranes, Mesosomes, or Both?

Mesosomes are often attached to cell wall septa, which in turn are believed to be the major sites of cell wall synthesis (32, 70, 77, 206). A possible prediction is that some or all PBCs might therefore be localized in the mesosomes. The evidence on this question is unfortunately both inadequate and conflicting.

Duerksen (58) reports that 95% of the label in cells of *Bacillus cereus* treated with radioactive penicillin was released upon conversion of the cells to protoplasts by treatment with lysozyme. Because mesosomes are released from the cell by such treatment, this finding would imply that the PBCs are located exclusively in the mesosomes. However, in the body of the paper, it appears that release of 58% of the label is a more typical value. Moreover, part of this material is dialyzable. It may be due to free penicillin trapped by the cell wall, hydrolysis of the penicilloyl-PBC bond, or proteolysis of the penicillin-PBC complex. The strongest statement that can be drawn from these findings is that a portion of the PBCs may be located in the mesosomes.

A similar conclusion was reached by Forsberg and Ward for distribution of the D-alanine carboxypeptidase of *Bacillus licheniformis* (80). Here, the specific activity of this enzyme, the major PBC in those bacilli examined, was 65% as great in mesosomes as in peripheral membranes.

### Do Organisms Have One or Multiple Proteins Which Bind Penicillin?

A major change in thinking about how penicillin acts on cells has been necessitated by the recent finding of multiple PBCs. The earliest evidence for multiple binding components was the suggestion of Mohberg and Johnson (157) that synnematin B (cephalosporin N) did not react with all the penicillin-binding sites of *S. aureus* which are sensitive to penicillin G. However, the evidence presented was weak. Competition experiments where radioactive penicillin G and nonradioactive synnematin B were added simultaneously indicated that a small portion of the penicillin sites (0.4 nmol/*g* of cells) were not subject to competition by

synnematin. Although the authors tried to exclude the possibility that this noncompetitive binding was due to penicillin degradation products, nonspecific binding still remains a probable explanation for their findings.

Much stronger evidence for multiple PBCs came by inference from the demonstration that the D-alanine carboxypeptidase of *B. subtilis* was distinct from the cephalothin killing site and was most likely not vital for cell metabolism (13). In growing cells, exposure to low levels of cephalothin rendered the cells osmotically fragile under conditions where at least 85% of the carboxypeptidase activity remained. Conversely, exposure of growing cells to 6-aminopenicillanic acid could inhibit the carboxypeptidase 95% under conditions in which the growth rate of the organism was altered by 10% at most. These results implied the existence of two binding components, a cephalothin-sensitive killing site (transpeptidase) and a relatively cephalothin-resistant carboxypeptidase. Whether separate proteins accounted for these sites or whether a single protein somehow was differentiated so as to exhibit these different behaviors was not determined.

Evidence for the former explanation, the existence of distinct proteins, was provided by Suginaka et al. (225). Column chromatography and isoelectric focusing of solubilized membranes to which radioactive penicillin had been bound revealed multiple peaks of radioactivity. As discussed by the authors, the interpretation of these multiple peaks is unclear. Although they could represent separate proteins, they might also be artifacts caused by variations in charge or state of aggregation of a single protein. Indeed, the purified D-alanine carboxypeptidase from *B. subtilis* was subsequently shown to give multiple peaks upon isoelectric focusing in the presence of detergent (J. N. Umbreit, PH.D. thesis, Harvard University, Cambridge, Mass., 1972). Nonetheless, in the case of *B. subtilis* membranes, greatly different sensitivities to cephalothin of the bands found upon isoelectric focusing strongly argued for the existence of at least two distinct PBCs. The D-alanine carboxypeptidase would be among the cephalothin-resistant bands; the killing site would presumably be among the cephalothin-sensitive bands.

Development of less ambiguous separation techniques greatly clarified the issue of multiple PBCs. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of solubilized membranes to which penicillin had been bound revealed multiple peaks—five in *B. subtilis*, three in *B. cereus* (or *B. stearothermophilus* (245)), and two in *S. aureus* (15) (Fig. 8, 9). For the *B. subtilis* components, the proportions, molecular weights, and kinetics of binding of penicillins and cephalosporins were determined (15). Component V, which accounted for 70% of the total penicillin bound, was identified as the D-alanine carboxypeptidase. The evidence was that (i) it bound penicillins and cephalosporins at the same rate as did the carboxypeptidase and (ii) that it had the same molecular weight as the purified carboxypeptidase (232). Three of the other components, I, II, and IV, were cephalothin sensitive. The rates of antibiotic binding of all three of these components were within a factor of three of that estimated for the penicillin killing site for the series of four beta-lactam

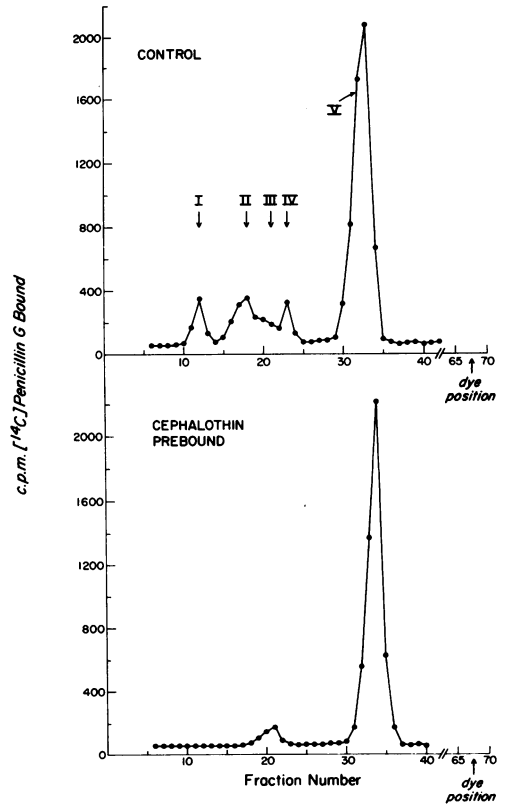



FIG. 8. *Penicillin-binding components of Bacillus subtilis. After [14C]penicillin G was bound, membranes were solubilized in sodium dodecyl sulfate (SDS) and subjected to SDS gel electrophoresis. Radioactivity in 1-mm gel slices is shown. Component V is the D-alanine carboxypeptidase. The failure of components III and V to react with cephalothin under the conditions used is shown below; prior treatment with cephalothin does not affect labeling of these components with [14C]penicillin G (15).*

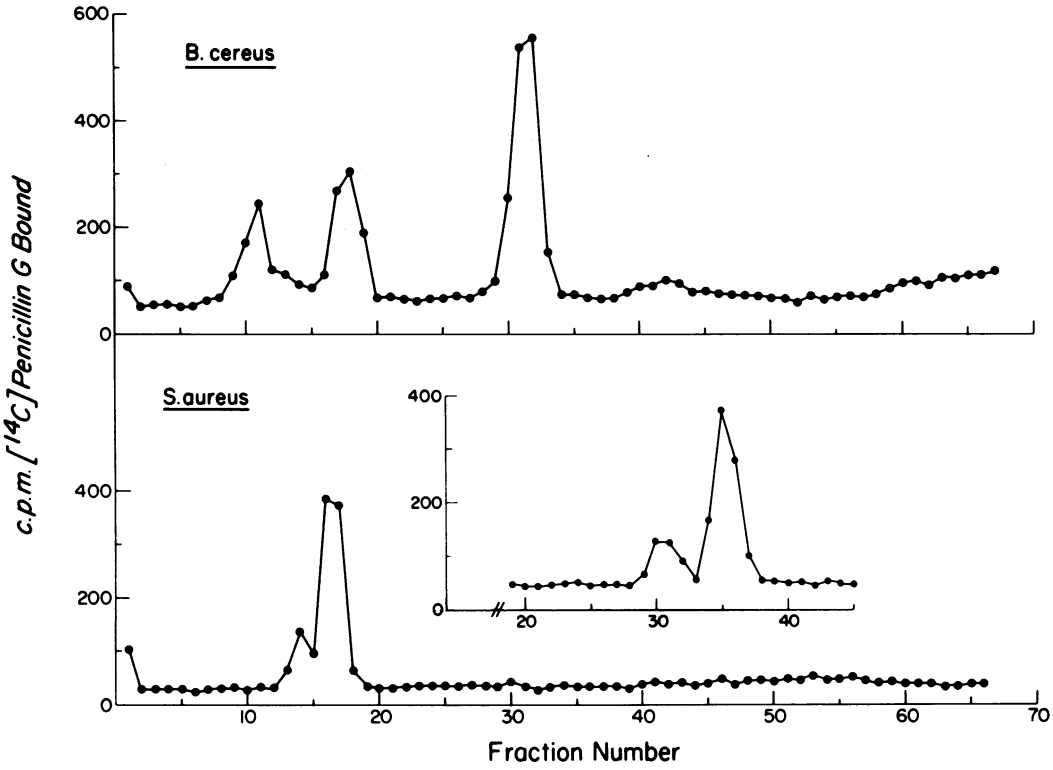



FIG. 9. *Penicillin-binding components of Bacillus cereus and Staphylococcus aureus. After binding of [$^{14}$C]penicillin G, membranes were solubilized in sodium dodecyl sulfate (SDS) and subjected to SDS gel electrophoresis (15).*

antibiotics examined. Consequently, all three are candidates for the penicillin killing site.

The multiple PBCs revealed by SDS gels did not appear to be artifacts of the gel procedure (15). Proteolysis was unlikely because the samples were boiled immediately after addition of the sodium dodecyl sulfate gel application buffer. Furthermore, treatment of the membranes with the protease inhibitor phenylmethane sulfonyl fluoride had no effect on the gel pattern. Aggregation caused by formation of disulfide bridges between proteins likewise seemed unlikely. Peaks eluted from gels and re-electrophoresed migrated with the same mobility as before, and the patterns obtained were quite reproducible from one experiment to the next. Further confidence in the validity of these results is provided by isolation of the PBCs by covalent affinity chromatography (14).

### Isolation of PBCs by Covalent Affinity Chromatography

The finding that neutral hydroxylamine could restore the activity of penicillin-inactivated D-alanine carboxypeptidase (115) and the development of convenient methods for coupling ligands to Sepharose (54) laid the stage for the isolation of PBCs by covalent affinity chromatography (14). The affinity resin was prepared by coupling 6-aminopenicillanic acid via its free amino group to a long succinyldiaminodipropylamine side chain attached to Sepharose. Membranes were solubilized with nonionic detergent and then reacted with the Sepharose-linked penicillin. After penicilloylation of the PBCs had occurred, the unbound proteins were eluted by extensive washing of the Sepharose in the presence of high salt. The PBCs could then be recovered free from contaminating protein by elution with hydroxylamine.

In the case of *B. subtilis*, the same pattern of five components was obtained by affinity chromatography as had been found earlier upon electrophoresis of whole membranes to which radioactive penicillin had been bound. Because protein rather than radioactivity was being monitored, the protein could be reduced prior to sodium dodecyl sulfate gel electrophoresis. This finding eliminated the possibility mentioned above that some of the binding components

observed might have been artifacts induced by formation of disulfide bridges.

Yields of the binding components were in general excellent. Carboxypeptidase was obtained in up to 80% yield. Those of components I, II, and IV were 50 to 110% of this. Component III, which bound penicillins quite poorly (15), was obtained in much poorer yield, approximately 10%. The PBCs did not appear to be denatured by the isolation procedure. All still bound pencillin. For all components, approximately one molecule of penicillin appeared to have been bound per molecule of protein (12).

Although the above procedure gave a mixture of five PBCs, a minor modification permitted purification of the *B. subtilis* carboxypeptidase (Fig. 10). If the membranes were treated with low levels of cephalothin before addition of the Sepharose-linked penicillin, binding to the affinity resin of the cephalothin-sensitive components I, II, and IV was blocked. Only components III and V were therefore contained in the hydroxylamine eluant from the affinity column. The customary low yield of component III in the affinity chromatography step, together with further loss during concentration of the eluant from the affinity column, reduced the level of contamination of the carboxypeptidase to less than 1%. Because the carboxypeptidase is a major membrane protein (15, 232), 0.75% of the total protein in the membrane, this procedure has now made available for study a PBC in quantities of the order of hundreds of milligrams. In addition, the procedure has also been applied for purifying the D-alanine carboxypeptidase from the thermophilic organism *B. stearothermophilus* (245).

A possible criticism of the demonstration of multiple PBCs in *B. subtilis* is that conceivably the multiplicity of components could have arisen from proteolysis of the binding component of highest molecular weight. Such a suggestion seems most unlikely. The proportions of the components are relatively constant. The rates at which they bind penicillins are dramatically different, up to four orders of magnitude. The carboxypeptidase, the component of lowest molecular weight, has a unique N-terminal amino acid. Nonetheless, although nonspecific proteolysis seems very unlikely, the possibility of specific proteolysis cannot be excluded. Proinsulin is converted to insulin, trypsinogen to trypsin. Such a process could be involved in control of cell wall synthesis. Comparison of the tryptic maps of the isolated binding components would resolve the matter in a straightforward fashion.

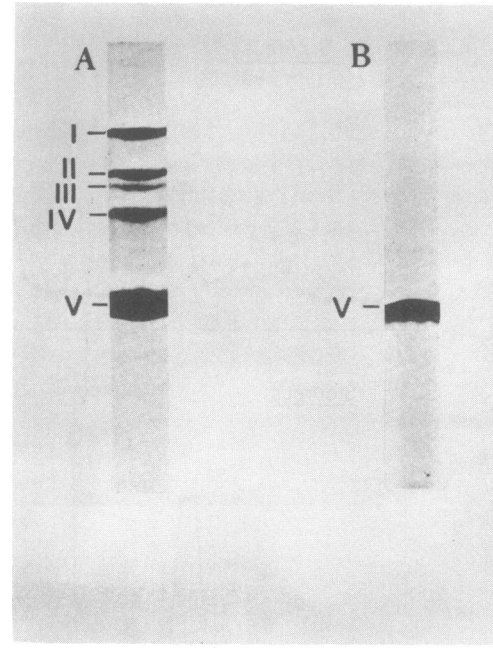



FIG. 10. *Penicillin-binding components of Bacillus subtilis isolated by covalent affinity chromatography. The isolated material was subjected to sodium dodecyl sulfate gel electrophoresis, then stained with Coomassie brilliant blue. A, The five penicillin binding components (compare with the radioactive trace in Fig. 8). B, Purified D-alanine carboxypeptidase (component V) prepared by the affinity chromatography method.*

Affinity chromatography on a cephalosporin C column has also been used as one step in a purification of the penicillinase from *B. licheniformis* (52). In that case, however, the enzyme is not covalently linked to the column and is eluted by a change in the pH of the buffer.

## What Is the Relationship of the PBCs to the Killing Site?

Tremendous effort has been devoted to study of the PBCs on the assumption that analysis of the PBCs may lead to a better understanding of the interaction of penicillin with its killing site. However, despite considerable effort, the relationship between PBC and killing site is still indeterminate. Basically, for a variety of wild-type organisms, the sensitivity to penicillins of the PBCs correlates with the sensitivity of the whole organisms. On the other hand, in penicillin-resistant mutants isolated in the laboratory, the resistance of the organisms is not reflected by the PBCs.

Early observations that penicillin-sensitive staphylococci bound penicillin whereas resist-

ant strains did not (50, 202) led to a closer examination of this problem. Eagle and colleagues (63, 64, 67) found that with four strains of cocci plus the rod-shaped *E. coli*, the amounts of penicillin bound at low penicillin concentrations were related to the sensitivity of the organism. The more sensitive organisms bound more. In addition, if all of the organisms were exposed to penicillin at the lethal dose $(LD)_{99.9}$ concentration, then the four cocci bound comparable amounts. The low binding by *E. coli* could be explained by the presence in that organism of penicillinase.

A different type of experiment carried out by Edwards and Park (69) correlated the sensitivity of the intact organism to penicillins and the sensitivity of the PBCs. They looked at the binding to one organism, *S. aureus* H, of a variety of penicillins and cephalosporins. Because the only radioactive penicillin commercially available is penicillin G, the procedure they used was first to bind the nonradioactive pencillin at different concentrations and then measure residual sites by reacting with labeled penicillin G. All the beta-lactam antibiotics examined saturated the penicillin G binding sites at approximately the minimal inhibitory concentration for the particular antibiotic.

These findings, together with those on the five binding components of *B. subtilis*, are consistent with the notion that some, but not all, PBCs may be targets for the killing action of penicillin. This evidence, however, is restricted to a narrow range of organisms, gram-positive cocci and a single gram-positive rod. No adequate analysis of gram-negative organisms or other gram-positive organisms has been made. Moreover, due to the presence of multiple PBCs, questions of correlation are only meaningful in terms of the correlation in sensitivity of the cell and a given PBC.

Examination of penicillin-resistant mutants derived in the laboratory (65, 66, 179) did not provide the expected support for the hypothesis that the PBCs were the target for the lethal action of penicillin. Although resistance was not due to an increased rate of inactivation of the penicillin, a good correlation between resistance and binding was not observed. For the four cocci examined by Eagle (65), one organism decreased the amount of penicillin it would bind while its penicillin resistance remained unaltered. Another increased its amount of binding when its penicillin resistance increased. This study, like much of the early work, was less informative than it would otherwise have been because the reaction of penicillin was analyzed in terms of total binding. The rate of saturation of PBCs by penicillin is the important factor, not whether the total amount of penicillin bound at saturation increases or decreases.

In a more recent study, mutants of *S. aureus* H resistant to up to $10^5$ times the penicillin concentration lethal for the parent strain were isolated (179). They still bound large amounts of penicillin at very low penicillin concentrations (0.1 μg/ml for some) although, in fact, the amounts were only 50% of that bound by the parent. The authors hypothesize an explanation for their data in terms of (i) "functional versus nonfunctional transpeptidase molecules," (ii) "variations in accessibility to penicillin," and (iii) substrate protection of transpeptidase.

A study by Sabath et al. (207) sheds further light on the problem. Strains of *S. aureus* $10^4$ times more resistant to methicillin than the wild type were isolated. The pH of the medium had a dramatic effect on this resistance. At pH 5.2 the resistance was not expressed. In contrast, pH had little effect on the sensitivity of the wild-type organism to the antibiotic. The interpretation is that this "intrinsic resistance" shown by the mutants is due not to an alteration in the target enzyme, but to a change in its accessibility. A similar situation may exist in the mutants isolated by Park et al. (179).

The conclusion that can be drawn from the above studies with mutants is that the situation is quite complex. The results can well be explained by the presence of multiple PBCs, only some of which are lethal targets for penicillin. In such cases, binding by the nonlethal target may obscure the relevant reaction. This possibility had in fact been considered as one explanation for the results (65). At that time, the techniques were not available to test the hypothesis. A second possibility is that some portion of the PBCs may be maintained in a cryptic state. If a cryptic site were the killing site and if penicillin binding to it could be observed only, for example, after disruption of the cells, then detection of alterations of it in mutant cells would not be observed by the techniques ordinarily used. A third possibility, which cannot be ignored, is that none of the PBCs may be the lethal target in some microorganisms. The case of *Bacillus megaterium* (see later section) may provide an example.

### Biochemical Evidence for the Involvement of PBCs in Peptidoglycan Synthesis

If PBCs are involved in cell wall synthesis, a prediction is that if cells are saturated with penicillin and the penicillin is then removed,

cell wall synthesis should reflect the blockage of the PBCs. Only two studies of limited scope have examined this question, both using the same organism, *S. aureus* strain H.

Rogers (197) examined the effect of prior binding of penicillin followed by removal on the *total amount* of peptidoglycan synthesized (he did not look at the degree of cross-linking). He found that prior reaction with penicillin greatly sensitized the amount of total cell wall synthesis to inhibition upon readdition of penicillin. He interpreted this and other results to indicate that most of the penicillin binding in *S. aureus* was not important, but that a small number of sometimes cryptic penicillin-sensitive sites played a vital role.

The second study (179, 180) showed that cells of *S. aureus* grown in the presence of penicillin showed subsequent inhibition of the total amount of cell wall synthesized. Moreover, although experimental details were not given, exposure of cells in buffer to penicillin followed by washing of the cells led to partial inhibition of cell wall cross-linking. This latter result would suggest that one of the PBCs is a transpeptidase in this organism.

In this sort of experiment direct and indirect effects of the penicillin treatment must be differentiated. If cells become "sick" during the penicillin treatment, the subsequent effect on cell wall synthesis may reflect this disturbance rather than the irreversible binding of the penicillin. Moreover, peptidoglycan synthesis and cross-linking are different phenomena; the role of the PBCs in these two processes may well be different. Finally, results in *S. aureus* cannot be generalized either to gram-positive bacilli or to gram-negative organisms.

## PENICILLIN-SENSITIVE ENZYMES

The above discussion deals with one approach to the study of the mode of action of penicillins, namely, the analysis of proteins which irreversibly bind beta-lactam antibiotics. The discovery that penicillin inhibited cell wall biosynthesis led to a second line of investigation in the study of the enzymatic activities involved in cell wall metabolism and their sensitivities to penicillins. As was the case with the PBCs, cell wall enzymology has turned out to be far more complex than had initially been believed. In all, three different penicillin-sensitive enzymatic activities have now been reported. Several major issues in the field at the present time can be defined. (i) What relationship do the different enzymatic activities have to each other? Are they catalyzed by the same or separate enzymes? (ii) How many proteins (enzymes) in a given organism catalyze any one of these activities? (iii) What is the function of the different types of penicillin-sensitive enzymes? (iv) What relationship do the different activities have to the mode of killing by penicillin; which one or ones are the killing site? (v) How does penicillin interact with these enzymes?

The different penicillin-sensitive enzymatic activities found in bacteria include (i) transpeptidases, assayed either with natural or with artificial acceptors; (ii) D-alanine carboxypeptidases; and (iii) endopeptidases, enzymes which cleave cross-linked peptide dimers of the peptidoglycan. Of these activities, the first is of particular interest because penicillin is thought to kill bacteria by inhibiting peptidoglycan cross-linking.

### Transpeptidase

**Transpeptidase assayed with natural acceptor in a coupled system.** This first assay most closely approximates the in vivo transpeptidation reaction. After addition of substrate, UDP-acetylmuramyl-pentapeptide, the reaction proceeds through the complex series of polymerization steps which culminate in peptide cross-linking and release of the terminal D-alanine from the pentapeptide chain (Fig. 3, 4, and 5). Although for many years such a system had only been demonstrable with the gram-negative organisms *E. coli* and *Salmonella* (2, 3, 105, 106), similar reactions have now been carried out with *B. megaterium* (239, 240), *Micrococcus luteus* (152), *S. aureus* (154), *Sporosarcinia ureae* (128), and *B. stearothermophilus* (127).

The course of the reaction can be monitored by release of the C-terminal D-alanine from the pentapeptide. The reaction product is applied to paper and chromatographed in isobutyric acid–1 N ammonia (5:3) to separate substrate, degradation products, newly formed peptidoglycan, and liberated D-alanine. Under optimal conditions, D-alanine release is coupled to peptidoglycan cross-linking; 1 mol of D-alanine is released per mole of acetylmuramyl-pentapeptide incorporated into the peptidoglycan. Analysis of the reaction products confirms the occurrence of cross-linking. Lysozyme digestion of the products leads to the release of dimers of disaccharide-peptide. In contrast, if cross-linking is prevented by penicillin, only monomers of the disaccharide-peptide can be obtained.

Several factors seem relatively important in obtaining an in vitro peptidoglycan synthesizing system of the sort described above. First of

all, simplicity in the cell wall structure is a major advantage. Because the coupled system requires the activity of all those enzymes functioning in the biosynthetic pathway beyond the level of the UDP-acetylmuramyl-pentapeptide, each modification of the pentapeptide which is required for eventual cross-linking reduces the chances of success. Thus, the two best-studied peptidoglycan-synthesizing systems are in *E. coli* and *B. megaterium*, neither of which has modifications of the basic pentapeptide structure in its peptidoglycan. Conversely, in *B. subtilis*, where the carboxyl group of the diaminopimelic acid residue of the pentapeptide is amidated (235), it has not been possible so far to demonstrate in vitro cross-linking, although synthesis of linear peptidoglycan can be obtained (115). The existence of modification reactions is not an insuperable barrier, however. Recently cross-linking has been demonstrated both in a system from *B. stearothermophilus* which includes an amidation reaction (127) and also in the even more complicated system in *S. ureae* (128), where the addition of glycine and D-glutamic acid in the cross bridge precedes cross-linking.

A second important factor is a fairly low level of autolytic and other degradative enzymes. For example, although autolysins cause only minor problems in assays with *E. coli*, they cause rapid breakdown of the newly formed peptidoglycan in *B. subtilis* (115). Degradation of the substrate must also be avoided. D-Alanine carboxypeptidases release the terminal D-alanine from UDP-acetylmuramyl-pentapeptide, rendering it incapable of acting as a donor in transpeptidation. As a further drawback, this D-alanine release by the carboxypeptidase can obscure the specific D-alanine release catalyzed by the transpeptidase. In such cases, the extent of transpeptidation must be monitored by the more laborious procedure of lysozyme digestion followed by analysis of dimer formation. Thus, transpeptidation is far more readily assayed in *E. coli* K-12, which has low levels of D-alanine carboxypeptidase, than in *E. coli* B, which possesses high levels (105, 106).

Thirdly, the gentle preparation of the appropriate membrane fraction is critical. Different means of breakage can affect specific activities by more than a factor of ten (193, 194, 239). Grinding with glass beads is apparently fairly gentle; rupture in a French press is more severe (193). Furthermore, the membrane fractions composed of larger membrane pieces appear to be more active (154, 193). Presumably, excessive fragmentation of the membrane leads to disorganization of the enzyme system. In accord with this general concept, complete peptidoglycan synthesis was obtained in what was essentially the cell wall fraction of *S. aureus* and *M. luteus* (152, 154). It was suggested that the membrane attached to the cell wall might be precisely that active at the growing points for new cell wall synthesis.

The different transpeptidase activities assayed by means of the coupled cross-linking reaction have all proved to be penicillin sensitive. However, although the *E. coli* (3, 105, 106), *S. aureus* (154), and *M. luteus* (152) systems are irreversibly inhibited by penicillins (i.e., the transpeptidases presumably are PBCs), this result cannot be generalized. The transpeptidase of the *B. megaterium* system is reported to be *reversibly* inhibited by penicillins (239, 240). In this organism, therefore, the transpeptidase may *not* be a PBC.

**Transpeptidase assayed with unnatural acceptor in a coupled system.** A major difficulty inherent in any coupled reaction as an assay for transpeptidase is that it cannot be used to assay fractionated membranes. Consequently, it cannot provide an assay useful for purification. From this point of view, transpeptidation reactions utilizing simpler unnatural acceptors are potentially of more value.

Two such reactions have been described. In *B. megaterium*, inclusion of a D-amino acid at moderately high concentration in the peptidoglycan synthesizing system leads to incorporation of the D-amino acid into the newly synthesized peptidoglycan (239, 240). Analysis of the products revealed that the D-amino acid (diaminopimelic acid was used customarily) was incorporated via a transpeptidation in the normal fashion. Because the enzyme recognized the D-center of the amino acid, dimer formation occurred in the presence of DD-diaminopimelic acid, which possesses two such centers. The products formed are shown in Fig. 11.

This system, although slightly simpler than the normal transpeptidation assay, is still a coupled system. The simultaneous presence of the diaminopimelic acid during synthesis of linear peptidoglycan is required. The activity is penicillin sensitive. Of the inhibition, about one-half is reversible. The rest is irreversible (see later section).

**Transpeptidase assayed in a simple, uncoupled system.** An important development has been the demonstration of transpeptidase reactions catalyzed by purified D-alanine carboxypeptidases from *Streptomyces* spp. (60, 89–92, 184, 186) and *B. stearothermophilus* (195, 245).

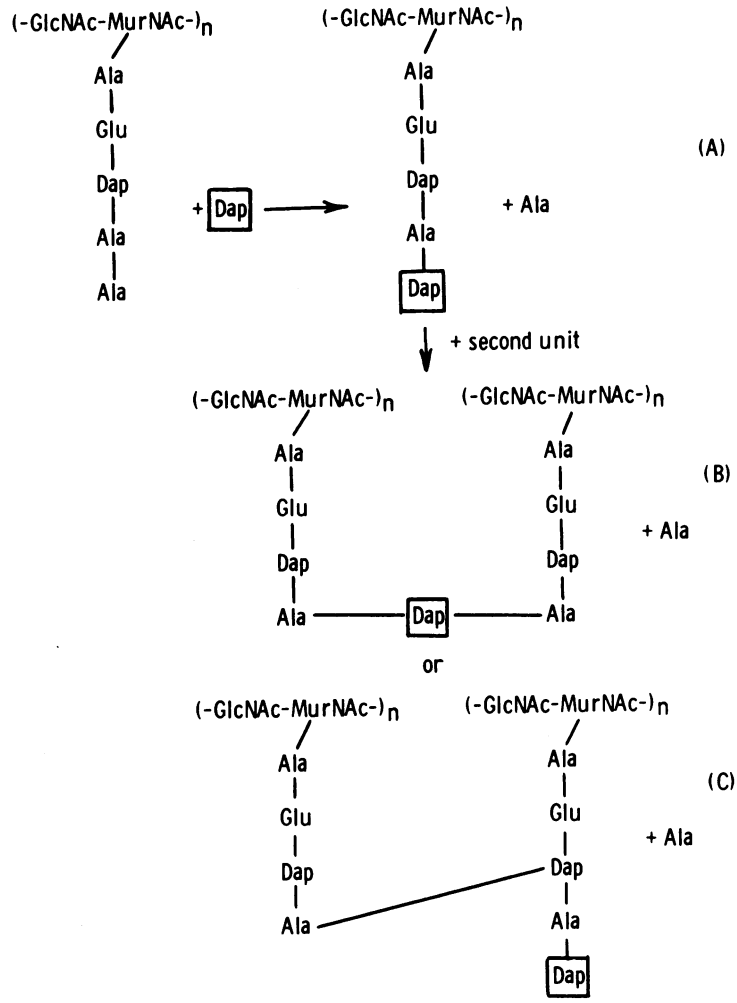



FIG. 11. *Transpeptidation reactions in particulate enzyme from Bacillus megaterium. Three separate transpeptidation reactions can occur. (A) Diaminopimelic acid (Dap) is incorporated at the end of the peptide subunit replacing* D*-alanine. Either meso- or* DD*-diaminopimelic acid can be utilized (but not the* LL *form). (B) If* DD*-diaminopimelic acid is the substrate in reaction one, a second subunit can interact with the first one, with the two amino groups of Dap forming a bridge between the two peptidoglycan units. (C) If meso-diaminopimelic acid is the substrate in reaction one, a different transpeptidation results in a normal cross-link between the two strands and a diaminopimelic acid terminal residue.*

These assays employ synthetic and natural peptides which function in an uncoupled system both as donors and acceptors in the reactions. These activities were discovered in enzymes which were first isolated as D-alanine carboxypeptidases and have given rise to extensive discussion of the physiological functions of such "DD-carboxypeptidase transpeptidases." They are discussed below together with the carboxypeptidases.

### D-Alanine Carboxypeptidase

In addition to the transpeptidases, a second penicillin-sensitive activity which has attracted considerable attention is that of the D-alanine carboxypeptidases, first encountered in *E. coli* (2, 105). These enzymes specifically hydrolyze the peptide bond between the two terminal D-alanine residues of UDP-acetylmuramyl-L-ala-D-glu-*meso*-Dap-D-ala-D-ala (Fig. 12). So far, carboxypeptidases which utilize the corresponding L-lysine-containing substrate have not been identified. Upon its discovery, it was postulated that the D-alanine carboxypeptidase might be an "uncoupled" transpeptidase (105, 115), where water rather than an amino group displaced the enzyme from the postulated acyl-enzyme intermediate (see Fig. 12). Other possi-

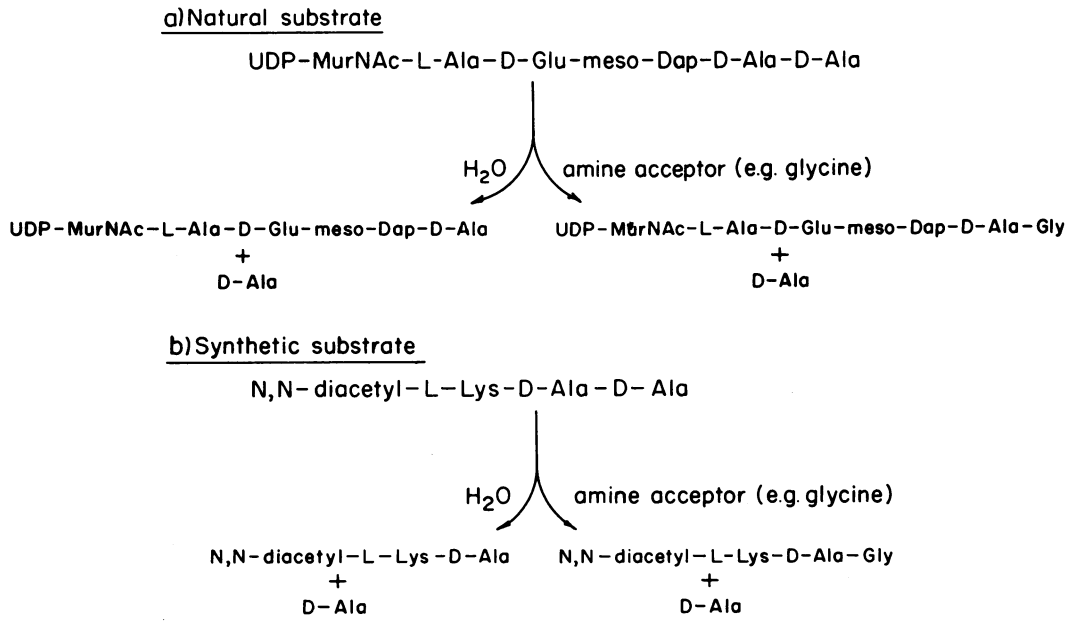



FIG. 12. *D-Alanine carboxypeptidase and model transpeptidase reactions.*

ble roles have also been suggested.

Three classes of carboxypeptidases have been examined. Some of their properties are summarized in Table 1. Because these enzymes are the only penicillin-sensitive enzymes which so far have been purified to homogeneity, they have been the object of most of the detailed study of the interaction of penicillin and substrates with penicillin-sensitive enzymes.

**E. coli carboxypeptidase.** The soluble *E. coli* carboxypeptidase has only been partially purified (107). Unlike the other carboxypeptidases, it may have a requirement for the nucleotide moiety of its substrate; the enzyme is only 20% as active on acetylmuramyl-pentapeptide as on UDP-acetylmuramyl-pentapeptide (107). This reduction in activity may or may not be an artifact of metal ion concentration. The enzyme also stands out for its sensitivity to penicillins. Penicillin G at a concentration of 0.002 μg/ml causes 50% inhibition.

**Carboxypeptidases from Bacillus.** The *B. subtilis* carboxypeptidsase by contrast is located in the membrane and has been purified to homogeneity by two procedures: by conventional enzyme purification techniques after solubilization in detergent (231, 232) and by covalent affinity chromatography (14). The enzyme has been extensively studied both as a PBC and as a penicillin-sensitive enzyme. Unlike the *E. coli* carboxypeptidase, it does not appear to recognize the nucleotide portion of its substrate, because it possesses comparable activity on UDP-acetylmuramyl-pentapeptide and acetylmuramyl-pentapeptide (115). In addition, the *B. subtilis* enzyme can cleave the synthetic substrate *N,N*-diacetyl-lysyl-D-alanyl-D-alanine used to assay the *Streptomyces* enzymes (see below). The *B. subtilis* carboxypeptidase displays intermediate sensitivity to penicillins. It is inhibited by 0.3 μg/ml of penicillin G (13). However, it is only inhibited by 150 μg/ml of cloxacillin.

The *B. stearothermophilus* carboxypeptidase resembles the *B. subtilis* enzyme in most respects. It is distinguished, however, by its unusual temperature stability. It should be noted that its purification by covalent affinity chromatography, at least as reported, is less satisfactory than that of the *B. subtilis* enzyme. The low increase in specific activity suggests that partial inactivation of the enzyme may occur during purification. Like the *Streptomyces* carboxypeptidases discussed below, the *B. stearothermophilus* enzyme can carry out a single transpeptidation reaction by using UDP-acetylmuramyl- L-ala- D-glu-*meso*-Dap- D-ala- D-ala as donor and glycine or D-alanine, but not diaminopimelic acid, as acceptor (11, 195) (Fig. 12). At an acceptor-donor ratio of 100:1, the percentage of donor transpeptidated was greater than 60%. Indeed, at a 1:1 ratio of D-alanine to donor, 5% transpeptidation still occurred.

**Streptomyces carboxypeptidases.** The *Streptomyces* spp. apparently secrete into the

TABLE 1. *Properties of D-alanine carboxypeptidases*

| Class | Organism | Location | Inhibition by penicillin | Mol wt | Transpeptidase activity | Ref |
|---|---|---|---|---|---|---|
| A | *Escherichia coli* | Membrane and cytoplasm | Reversible (?), highly sensitive | | + (?) | 85, 105–107, 183[a] |
| | *Anabaena variabilis* | | | | | |
| B | *Bacillus subtilis* | Membrane | Irreversible | 50,000 | - | 13–15, 115, 231–234 |
| | *Bacillus stearothermophilus* | | | 46,500 | + | 11, 16, 193, 195, 245 |
| | *Bacillus megaterium* | | | | ND[b] | 193, 239, 240 |
| C | *Streptomyces* | Culture medium | Reversible or insensitive | 37,000, 55,000 | +, Some strains only | 88, 92, 120–123, 161, 164 |

[a] See also J. J. Pollock, M. Disteche, R. Linder, M. R. J. Salton, and J. M. Ghuysen, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, P77, p. 153.
[b] ND, Not determined.

culture medium enzymes possessing D-alanine carboxypeptidase activity on the synthetic substrate *N*,*N*-diacetyl-lysyl-D-alanyl-D-alanine (88, 120–123). Three of the four enzymes examined (from four different *Streptomyces* species) are also capable of acting as transpeptidases by using as donor *N*,*N*-diacetyl-L-lysyl-D-alanyl-D-alanine and as acceptor D-alanine, glycine, or diaminopimelic acid. Many interesting observations have been made during study of these enzymes. Their properties are summarized in Table 2.

Determination of carboxypeptidase activity on an extensive series of synthetic peptides of the general structure X – $R_3$ – $R_2$ – $R_1$ (OH) reveals certain substrate requirements of the enzymes (120, 121, 123):

(i) The C-terminal amino acid ($R_1$) should be a free acid, although there is some activity with the amide. This residue must be either glycine or else a D-amino acid. The specificity for the D-amino acid is relatively low.

(ii) The $R_2$ residue should be D-alanine. There is some activity with glycine, but none with other D- or L-amino acids. The strictest specificity occurs at this residue.

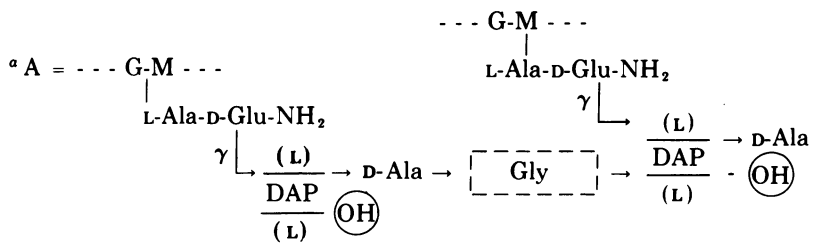
(iii) The $R_3$ residue must be an L-amino acid and substituted on its $\alpha$-amino group, i.e., X cannot be a hydrogen atom. It should also possess a long side chain. Thus, $R_3$ cannot be hydrogen or acetyl, i.e., D-alanyl-D-alanine and acetyl-D-alanyl-D-alanine are not substrates. For some strains, namely *Streptomyces albus* G, K11, and R61, any additional terminal amino group on the side chain of $R_3$ (e.g., on lysine) should be acylated. For strain G, but not R61, the enzyme is also active if this amino group is in an $\alpha$ position to a free carboxyl group (e.g., as in diaminopimelic acid). In contrast, with the R39 enzyme, acylation of the amino group reduces the activity. For all four enzymes, substitution of the $\epsilon$-amino group of lysine at $R_3$ with pentaglycine gives a highly active substrate.

The enzymatic efficiency of the four carboxypeptidases on the different substrates is a function both of the Michaelis constant ($K_m$), for binding the substrate, and the catalytic constant $V_{max}$. In the case of strains R39 and albus G, good substrates bind to the enzyme with lower $K_m$ than do poor substrates. The values for $V_{max}$ are less affected. For strains R61 and K11, the situation is reversed. All the substrates bind with high $K_m$s; the good substrates are distinguished by having much greater values of $V_{max}$ (123). These results are interpreted to indicate that the binding surface

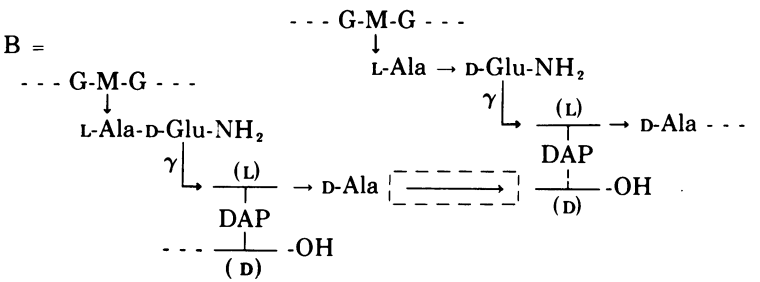
TABLE 2. "*DD-Carboxypeptidase-transpeptidase*" *from Streptomyces*

| Strain | Ref | Mol wt | Isoelectric point | Penicillin sensitivity | Cell wall structure[a] | Kinetics of penicillin inhibition | Lytic on cell walls | $K_m$ for good substrates | Transpeptidation demonstrated |
|---|---|---|---|---|---|---|---|---|---|
| G | 87, 88, 121, 122 | | >8.5 | − | A | | Yes | Low | No |
| R61 | 81, 82, 92, 120 | 38,000 | 4.8 | + | A | Competitive | No | High | Yes |
| K11 | 123 | | <8.5 | + | A | Competitive | No | High | Yes |
| R39 | 89, 123 | 55,000 | <8.5 | + | B | Partially competitive (?) | No | Low | Yes |

[a] A =




B =



for the R61 and K11 enzymes is relatively nonspecific, with good substrates inducing a conformational change leading to catalytic activity. Conversely, the R39 and albus G enzymes would have a much more specific binding surface. Actually, as shown in Table 3, these differences in the range of $K_m$ and $V_{max}$ for the different enzymes are not always too great.

Peptide analogues of the *N*,*N*-diacetyl-L-lysyl-D-alanyl-D-alanine function in some cases as competitive inhibitors of the albus G and R61 enzymes (164). Such studies suggest that the two C-terminal residues of the tripeptide function to promote binding to the enzyme. The third residue has little effect on binding. Rather, the side chain is crucial for promoting catalytic activity. In addition, it has been suggested that the normal configuration of the peptide bond in the C-terminal dipeptide during binding to the enzyme is *cis*. This conclusion is based on one observation: that the acetyl-racemic-cyclodiaminoadipic acid lactam acts as a relatively good inhibitor of the R61 and albus G enzymes. This compound is an alanyl-alanine analogue, constrained to a *cis* configuration by its ring formation. These conclusions ought not be generalized to the R39 enzyme. This enzyme was not inhibited by any of the peptide inhibitors examined; indeed, some of the peptides were good substrates for the enzyme.

(i) Transpeptidase activity with amino acid acceptors: In addition to D-alanine carboxypeptidase activity, the R39, R61, and K11 enzymes, but not the albus G enzyme, also possess transpeptidase activity. This activity was first shown by using *N*,*N*-diacetyl-L-lysyl-D-alanyl-D-alanine as donor and D-alanine or glycine as acceptor (186). In the presence of acceptor, three reactions occurred. Donor was cleaved by the carboxypeptidase activity, donor was transferred to acceptor, and the transpeptidation product was cleaved by the carboxypeptidase activity. The proportion of donor converted to transpeptidation product under optimal conditions was quite high, greater than 60% for either R61 or R39 at an acceptor to donor ratio of 100:1 (i.e., 0.15 M acceptor). At lower acceptor-donor ratios, the amount of transpeptidation which occurred was still substantial: At 10:1, it was 55 and 40% for the R61 and R39 enzymes, respectively. Indeed, 5% transpeptidation still occurred with the R61 enzyme at a 1:125 ratio.

Several factors affected the relative activities of the enzymes as transpeptidases and carboxypeptidases. High pH favored transpeptidation (82, 89, 92). Likewise, a 50% reduction in water content in the assay mixture by ethylene glycol and glycerol (65:35, vol/vol) decreased hydrolysis of donor tripeptide considerably, from 60 to 25%, while having little effect on transpeptidation. In addition, as will be discussed below, high concentrations of transpeptidation acceptor not only enhanced transpeptidation but often decreased hydrolysis of donor by a greater amount than could be explained by competition between hydrolysis and transpeptidation.

(ii) Transpeptidase activity with dipeptide acceptors: The structure of the cell wall of *Streptomyces* strains R61 and K11 differs from that of strain R39 (which indeed suggests that strain R39 may have been improperly classified as *Streptomyces* [89]). Whereas the cross-bridge in the former strains extends through LL-diaminopimelic acid and glycine, that in the latter is a direct linkage via *meso*-diaminopimelic acid (see Table 2). In accord with this difference in cell wall structure, the acceptor specificity for the R61 and K11 enzymes differs from that of the R39 enzyme.

Although both the R39 and R61 enzymes could use as acceptor the amino acids glycine, D-alanine, and *meso*-diaminopimelic acid, only the R61 enzyme was active with a wide range of compounds (184). Dipeptides with N-terminal glycine were most active. Those with N-terminal D-alanine were less so. Good acceptors had a

TABLE 3. *Catalytic efficiencies on different substrates of DD-carboxypeptidases from Streptomyces*[a]

| Expt and strain | $K_m$ (mM) | Range of $K_m$ | $V_{max}$ (μmol/mg per h) | Range of $V_{max}$ | Ref |
|---|---|---|---|---|---|
| Expt 1 | | | | | |
| Albus G | 0.3–15 | 50 | 9–100 | 11 | 120 |
| R61 | 10–36 | 3.6 | 1.7–890 | 520 | 120 |
| Expt 2 | | | | | |
| R39 | 0.2–2.4 | 12 | 40–3,000 | 75 | 123 |
| K11 | 8–30 | 3.8 | 9–2,000 | 222 | 123 |

[a] Different sets of substrates were employed in the two experiments, so comparison between experiments is not justified.

C-terminal amino acid which neither was too bulky nor possessed a D-asymmetric center in the group involved in the peptide bond. Thus, the best acceptors were either glycyl-L-alanine, glycyl-glycine, or $\epsilon$-glycyl-L-lysine. Perhaps a little surprisingly, $\alpha$-glycyl-$\alpha'$-acetyl-LL-diaminopimelic acid, the analogue of the natural acceptor in the cell wall, gave four- to fivefold less transpeptidation than the above acceptors. A large number of other acceptors also functioned with the R61 enzyme. These included the lactam of *meso*-diaminopimelic acid, 2-amino-2-deoxyhexuronic acid, D-cycloserine, and 6-aminopenicillanic acid (a poor acceptor).

(iii) Transpeptidase activity with cell wall peptides as acceptors: The use of natural cell wall peptides as acceptors for the R61 enzyme has not been reported. However, although dipeptides did not function as acceptors for the R39 enzyme, analogues of the natural cell wall peptide acceptor were active (89). Here, specificity was directed at the tripeptide sequence L-alanyl-$\gamma$-D-glutamyl-(L)-*meso*-diaminopimelic acid. Substitution either of the N-terminus with the disaccharide $\beta$-1,4-*N*-acetyl-glucosaminyl-*N*-acetylmuramic acid or else of the C-terminus with D-alanine had little effect. In contrast, amidation of the $\alpha$-carboxyl on the glutamic acid or of the D-carboxyl on the diaminopimelic acid residues had profound effects. The latter substitution rendered the compound completely inactive. The former converted it to the natural cell wall tetrapeptide in this organism and caused major changes in its influence on hydrolysis and transpeptidation. Transpeptidation by using natural amidated donor as well as acceptor has not yet been reported. However, at high concentrations of peptide, the R39 enzyme does catalyze dimer formation between two molecules of the pentapeptide L-alanyl-$\gamma$-D-glutamyl-L-*meso*-diaminopimelyl-D-alanyl-D-alanine (92). This peptide differs from the one naturally found in the wall only in its lack of amide substitution on the $\alpha$-carboxyl of the glutamic acid.

Multiple observations indicated that the nature and concentration of the acceptor affected the efficiency of the R39 enzyme both as a carboxypeptidase and as a transpeptidase (89). (i) With L-alanyl-$\gamma$-D-glutamyl-(L)-*meso*-diaminopimelic acid as acceptor, increasing the concentration of acceptor above a 9:1 acceptor to donor ratio had no further effect on the extent of transpeptidation. It did, however, considerably decrease the extent of donor hydrolysis. (ii) Amide substitution of the $\alpha$-carboxyl of the glutamic acid in the above tripeptide acceptor caused transpeptidation to occur only within a narrow range of acceptor-donor ratios (1 to 3:1 was optimal, depending on the donor concentration used). Higher ratios strongly inhibited both transpeptidation and hydrolysis. (iii) The semiamidated peptide dimer (Fig. 13) did not itself serve as an acceptor. However, it strongly inhibited both the carboxypeptidase activity of the R39 enzyme and also its transpeptidase activity with glutamic-amidated tetrapeptide as acceptor.

Basically similar although less extensive evidence exists for such effects by acceptor on the R61 enzyme (82, 184). High concentrations of dipeptide acceptor inhibited both transpeptidation and donor hydrolysis. The relative sensitivity of the two processes depended on the specific acceptor examined. When diaminopimelic acid was used as acceptor instead, no such inhibition occurred.

In addition to the purified soluble DD-carboxypeptidase-transpeptidase discussed above, *Streptomyces* R61 also possesses a similar membrane-bound transpeptidase activity (60, 92). This activity differs from that of the soluble enzyme in that in the membrane it acts exclusively as a transpeptidase. Carboxypeptidase activity appears, however, upon solubilization of the activity in 2 M urea, 10 mM ethylenediaminetetraacetic acid. In addition, the membrane activity differs from that of the soluble enzyme in the efficiency of transpeptidation obtained with different acceptors, being more efficient with some substrates and less efficient with others.

## Endopeptidase

Several endopeptidases have been found in *E. coli* (17, 98, 183). These enzymes cleave the disaccharide-tetrapeptide dimer between the D-alanine and the diaminopimelic acid residues

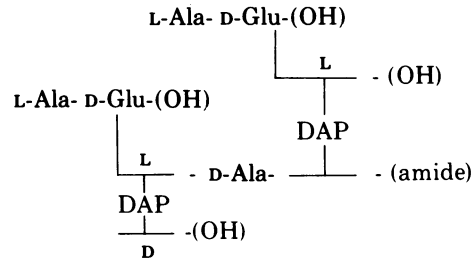



FIG. 13. *Structure of the semiamidated peptide affecting the activity of the DD-carboxypeptidase-transpeptidase from Streptomyces strain R39.*

(Fig. 14). Such endopeptidases thus act as autolytic enzymes which antagonize the action of the transpeptidase. At least one endopeptidase activity in *E. coli* is penicillin sensitive (17); a second activity unaffected by the antibiotic has also been reported (98).

It has been noted that the endopeptidase substrate formally resembles that of a D-alanine carboxypeptidase, in that both possess a free carboxyl group in a position alpha to the peptide bond which is cleaved. The free COOH group in the cross-linked dimer is that on the D-asymmetric center of the diaminopimelic acid residue (Fig. 14). In agreement with this resemblance in substrate, the *B. stearothermophilus* (245), *Streptomyces* (82, 120, 123), and *E. coli* (17) carboxypeptidases also possess endopeptidase activity. The levels of activity as endopeptidases are less than as carboxypeptidases, however. The relative enzymatic efficiencies range from 17 to 0.15%.

## Other Activities

The presence in *E. coli* of a "penicillin sensitive glycosidase" was also reported (98; J. V. Höltje, Ph.D. thesis, Eberhard-Karls Universität, Tübingen, Federal Republic of Germany, 1970). However, the degree of sensitivity of this enzyme to penicillin is not very great. The data show that penicillin at 1,000 U/ml (600 μg/ml) inhibits 20%; at 2,500 U/ml (1,500 μg/ml), it inhibits 40%. These values are sufficiently high so that the specificity of the reaction with penicillin is questionable. Because the enzyme apparently is very labile, bovine serum albumin is added for stabilization. The authors attempt to explain the relative insensitivity of the enzyme to penicillin in terms of a nonspecific interference with the action of penicillin by the bovine serum albumin. However, the validity of this explanation is dubious.

*Halobacterium salinarium* lacks the peptidoglycan characteristic of normal bacteria (112). Nevertheless, the organism has been reported to be sensitive to relatively high concentrations of penicillin (158). This finding suggests that penicillin may be able, at high concentrations, to inhibit some activity other than those with which it is normally associated.

## Number of Enzymes Corresponding to the Different Penicillin-Sensitive Enzymatic Activities

Many organisms are now known to contain several different penicillin-sensitive activities. *E. coli* has three: (i) transpeptidase assayed in a coupled, cross-linking reaction with natural acceptor, (ii) D-alanine carboxypeptidase, and

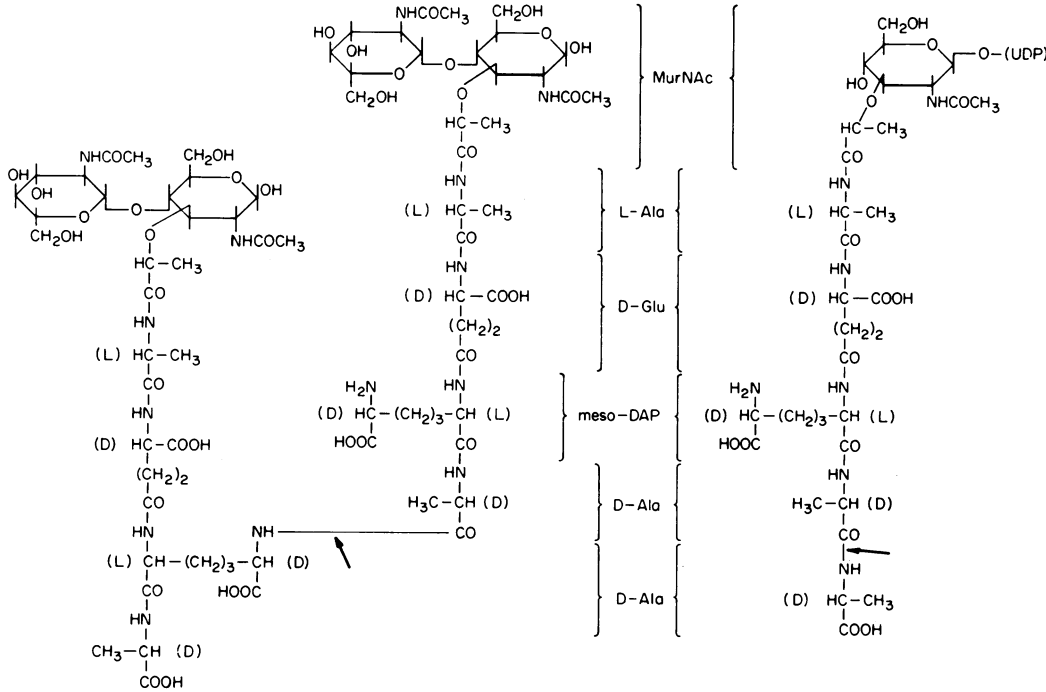



FIG. 14. *Comparison of substrates for endopeptidase and D-alanine carboxypeptidase.*

(iii) endopeptidase. In *B. megaterium*, likewise, three different activities are present: (i) transpeptidase assayed in a coupled cross-linking reaction with natural acceptor, (ii) transpeptidase assayed in a coupled system with unnatural acceptor, and (iii) D-alanine carboxypeptidase. The presence of these multiple activities emphasizes the potential complexity in the interaction of penicillin with the bacterial cell. They further raise the important question of the number of proteins which are responsible for the different activities. Resolution of this question is hampered by the small number of these enzymes which so far have been purified. One approach consequently has been to compare the response to penicillins of the different activities in question. Two questions have been examined: is the inhibition reversible, and what is the profile of sensitivity to different beta-lactam antibiotics?

By these criteria, *B. megaterium* would appear to have at least five separate enzymes (Table 4). The transpeptidase activity assayed by cross-linking with natural acceptors (Table 4) may be the product of two enzymes, because 30% of the activity was resistant to concentrations of cloxacillin ranging from 0.1 to 2.5 μg/ml. Likewise, the transpeptidase activity assayed with diaminopimelic acid as acceptor (Table 4) may be the product of two separate enzymes, because one-half of the activity is inhibited irreversibly by cloxacillin. Similarly, two D-alanine carboxypeptidases are present. The one active on the unnatural lysine-containing substrate is penicillin insensitive. The enzyme active on the natural substrate is penicillin sensitive. The data do not indicate, however, whether this latter activity could be the same as the cloxacillin-resistant portion of transpeptidase assayed with natural acceptor (Table 4).

In the *S. aureus* transpeptidase system (154), transpeptidation is inhibited 60% by penicillin G at 0.5 μg/ml. The remaining synthesis is considerably more refractory. Half-inhibition requires perhaps 25 μg/ml. This result might imply two separate transpeptidases in this organism (possibly corresponding to the two PBCs in *S. aureus*).

In *E. coli*, the transpeptidase is irreversibly inhibited (106, 107). Moreover, the sensitivity of the carboxypeptidase to penicillins is at least 100 times greater than is that of the transpeptidase. These enzymes therefore are presumably different. Carboxypeptidase and endopeptidase in *E. coli* have been suggested to be the same on the basis of their simultaneous presence in a partially purified carboxypeptidase preparation (17). Further purification, however, suggests that the situation is more complex. Multiple activity peaks were found on column chromatography. At least three types of enzyme were present, having (i) carboxypeptidase activity only, (ii) endopeptidase activity only, and (iii) both types of activity (224).

In *Streptomyces*, as discussed elsewhere, the soluble and membrane-bound transpeptidases probably differ. Among other evidence, the two enzymes show dramatically different profiles of sensitivity to various penicillins (60, 90). Likewise, the D-alanine carboxypeptidase of *B. subtilis* appears to differ from the penicillin killing site, presumably the transpeptidase (13).

TABLE 4. *Transpeptidase and D-alanine carboxypeptidase activities in B. megaterium membranes*[a]

| Determination | Inhibition by cloxacillin | Reversibility of inhibition by cloxacillin | Inhibition by penicillin G | Inhibition by cepholathin |
|---|---|---|---|---|
| Transpeptidase | | | | |
| Cross-linking with natural acceptors | 70% sensitive at 0.1 μg/ml | Yes | ND | ++ |
| | 30% resistant to 2.5 μg/ml | ND | ND | |
| Diaminopimelic acid as acceptor | 88% at 0.5 μg/ml | 50% | 100% at 3,000 μg/ml[b] | + |
| Carboxypeptidase | | | | |
| Assayed on natural substrate[c] | 50% at 500 μg/ml | No | 100 μg/ml | ND |
| Assayed on lysine-containing substrate | Insensitive | | Insensitive | Insensitive |

[a] Data are from reference 240; ND, not determined.

[b] Due to the presence of penicillinase in the membrane preparation, high concentrations of penicillin G were required to block these reversibly inhibited activities.

[c] The natural substrate was UDP-acetylmuramyl-L-ala-D-glu-*meso*-Dap-D-ala-D-ala. In the lysine-containing substrate, diaminopimelic acid was replaced by L-lysine.

On the other hand, multiple activities are possessed by several purified enzymes. The purified *Streptomyces* R61, K11, and R39 carboxypeptidases (82, 89, 92, 184, 186) and the purified *B. stearothermophilus* carboxypeptidase (245) also exhibit "transpeptidase" and endopeptidase activity.

Much of the above evidence for the existence of multiple enzymes is susceptible to different interpretations. In cases where activities are partially inhibitable, the assignment of multiple enzymes is least convincing. Because the assays are customarily performed on heterogeneous membrane preparations, differences in the location of the enzyme in the membrane could result in shielding of a certain proportion of the sites from penicillin. Likewise, artifacts in the determination of penicillin inhibition could generate the appearance of separate enzymes (see later section). Moreover, if penicillin acted as an allosteric inhibitor, complete binding might result in only partial inhibition of a homogeneous enzyme.

Another consideration is that some change in state or structure of one enzyme with one pattern of antibiotic sensitivities might lead to a new activity with a new pattern of interaction with penicillins. If the active site of a transpeptidase became accessible to water, it might be converted to a carboxypeptidase. The kinetics of penicillin inhibition might also be altered. Such an effect has been assumed (60, 92) to account for the difference in penicillin sensitivities of the soluble and particulate transpeptidases of *Streptomyces*. However, this hypothesis remains unproven.

To the contrary, the purified *B. subtilis* carboxypeptidase closely resembles the membrane-bound, unpurified enzyme; penicillin sensitivities for the enzyme in the two states agree with each other within a factor of two (233). A possibly more significant type of alteration could be proteolytic degradation. Because the molecular weight of the *B. subtilis* carboxypeptidase is only 40% of that of the PBC of highest molecular weight (I), removal of 60% of component I could easily change the protein sufficiently to account for the difference of up to $10^4$ in the reactivities to various penicillin derivatives.

Consequently, separate important questions are: (i) How many distinct proteins account for the assayable activities? (ii) How many genes code for these distinct proteins? The answer to the second question will require mutants defective in the different enzymes or structural comparison of the purified, distinct proteins.

## Relationship of the Penicillin-Sensitive Enzymes to the PBCs

The demonstration of multiple penicillin-sensitive activities is in accord with the existence of multiple PBCs. Both lines of evidence support the general conclusion that at least some of the multiple enzymatic activities are real rather than artifacts of assay. *S. aureus* may have two distinguishable transpeptidase activities. It likewise has two binding components. *B. subtilis* and *B. stearothermophilus* have cephalothin-sensitive killing sites and a cephalothin-resistant carboxypeptidase; they similarly possess both cephalothin-sensitive and cephalothin-resistant PBCs.

The binding components of *E. coli* have not been thoroughly investigated. However, in unpublished experiments at least two have been shown to exist. PBCs have not been analyzed in *Streptomyces*.

The demonstration in *B. megaterium* that the cross-linking activity is reversibly inhibited by penicillins while some other activities are irreversibly inhibited is very important. It emphasizes that the penicillin killing site may not be a PBC. Furthermore, it underlines the dangers in generalizing results from one class of bacteria to another.

## Physiological Functions of the Penicillin-Sensitive Enzymes

The physiological role of no penicillin-sensitive enzyme has been determined in a convincing fashion. The most thorough study has been devoted to the carboxypeptidases. The cell walls of organisms possessing carboxypeptidases show a relatively low degree of cross-linking. The peptidoglycan of *E. coli* or *B. subtilis* is cross-linked to form dimers or trimers at most. In contrast, the peptidoglycan of an organism such as *S. aureus*, which lacks a carboxypeptidase, is much more highly cross-linked. Because the product of the carboxypeptidase reaction is incapable of being a donor in cell wall cross-linking, the function of the enzyme may be to regulate the degree of linkage in the wall. An alternative hypothesis is that the carboxypeptidase activity is a side reaction of the transpeptidase.

Evidence against this latter hypothesis is the demonstration that cephalothin kills *B. subtilis* without inhibiting carboxypeptidase. Conversely, at least 95% of the *B. subtilis* carboxypeptidase could be inhibited without killing the cells. On the other hand, although inhibition of the carboxypeptidase was predicted to increase

the degree of cross-linking of the peptidoglycan, this has not been verified. Within the accuracy of the experiments (±7%), no change in cross-linking was observed at concentrations of 6-aminopenicillanic acid which caused up to 95% inhibition of the carboxypeptidase (213). Several factors may account for these results. (i) Five percent of the carboxypeptidase activity may be sufficient for the cell. This enzyme is present, after all, in much larger quantities in *B. subtilis* than are the putative transpeptidases. (ii) Only a small fraction of the total carboxypeptidase may function in cell wall synthesis. After residing only momentarily at the site of peptidoglycan synthesis, the enzyme might be displaced and become functionally inactive. Because the resistance of the enzyme to penicillins should be inversely proportional to its half-life (13), the physiologically relevent carboxypeptidase would be much more resistant than predicted. (iii) This putative small fraction of carboxypeptidase at the growth zone might be inaccessible to the penicillin and thereby protected.

Technically, the accuracy of the experiment suffers from the use of dinitrophenylation of unblocked amino groups on the diaminopimelic acid to determine the degree of cross-linking. If the effects on cross-linking were small, they might have been obscured by experimental error. More accurate determination should be possible with a recently developed technique, deamination of the free amino groups by nitrous acid (78, 153).

In *E. coli*, the carboxypeptidase is reversibly inhibited by penicillins. Consequently, although the $K_i$ for the enzyme is considerably below the concentration required to kill the cells, inhibition of the enzyme in intact cells cannot be verified. Indeed, the enzyme may not be accessible to penicillin. Much of it is cytoplasmic, and it is not clear either that penicillin penetrates the cytoplasmic membrane or that it does not suffer interference from the high protein concentration in the cytoplasm.

The properties of the *E. coli* enzymes support the suggestion that the carboxypeptidase could regulate cross-linking. The enzyme is five times more active on the soluble precursor UDP-acetylmuramyl-pentapeptide than it is on the acetylmuramyl-pentapeptide alone (107). On uncross-linked cell walls, even less activity could be shown. Moreover, it has been shown that the UDP-acetylmuramyl-tetrapeptide which results from the action of the carboxypeptidase can be incorporated into linear peptidoglycan (106). Experimental questions which would be of interest to answer are (i) whether UDP-acetylmuramyl-tetrapeptide is present in appreciable amounts in *E. coli* and (ii) whether the ratio of UDP-acetylmuramyl-tetrapeptide to UDP-acetylmuramyl-pentapeptide is affected by penicillin.

The membrane-bound transpeptidase assayable in *Streptomyces* R61 (60, 92) is quite plausibly a killing site for penicillin. The enzyme and organism differ by no more than a factor of five in their sensitivities to penicillin. Whether other potential lethal targets for penicillin exist in this organism is not known.

The role of the soluble DD-carboxypeptidase-(endopeptidase)-transpeptidase in *Streptomyces* is more of a puzzle. It is excreted into the medium and, unlike the case of the membrane-bound transpeptidase, there is no correlation between its sensitivity to different penicillins in strains R39, R61, and K11 and that of the organism. Moreover, whereas certain peptide analogues of the substrate inhibit this enzyme, they are not inhibitory for the organism (164). Likewise, no transpeptidase activity can be demonstrated in one of the "DD-carboxypeptidase-transpeptidases," that from *Streptomyces albus* G. Consequently, the soluble carboxypeptidase itself would appear to be distinct from the physiological transpeptidase.

The question arises, however, whether this enzyme could be a solubilized form of the membrane-bound transpeptidase. Inadequate evidence exists to resolve this question, although it has been stated without qualification that the two enzymes are related (60). The strongest evidence against the hypothesis is the disparity in penicillin sensitivities of the two enzymes (see preceding section). Likewise, the enzymes differ somewhat in the extent of transpeptidation they can perform with different acceptors (see earlier section). On the other hand, the membrane-bound transpeptidase upon solubilization of 2 M urea acquired some carboxypeptidase activity. Moreover, the specificity for acceptors, particularly for the R39 enzyme and the kinetics of inhibition of transpeptidation at high acceptor concentrations (82, 92) strongly suggest that the carboxypeptidases possess an acceptor binding site.

Most known membrane enzymes, except for those which have been solubilized by proteolysis, are water soluble only in the presence of detergent. If the membrane-bound transpeptidase of *Streptomyces* is related to the soluble carboxypeptidase-transpeptidase found in the culture medium of these organisms, it seems a strong possibility that the latter may be a

proteolytic fragment of the former. This possibility is perhaps strengthened by the fact that maximal activity in the culture filtrates from which the carboxypeptidases were prepared was obtained only after 50 to 135 h of incubation (87, 120, 123). An important, related question is whether the release of these enzymes from the cells is by chance or whether it is the result of a specific process. If it is a specific process, then what physiological function does the enzyme have for the organism?

The presence of a binding site for acceptor on the enzyme would be compatible either with a proteolytic origin for these enzymes or with an evolutionary origin from a transpeptidase gene. Alternatively, if the physiological function of the enzyme is neither as transpeptidase nor as carboxypeptidase, but rather as endopeptidase, then it would need to possess binding sites for both the "donor" and "acceptor" portions of the cross-linked dimer substrate. Studies of the carboxypeptidase from *Streptomyces* have already contributed to our knowledge of enzymes of cell wall metabolism, and resolution of the questions raised surely will significantly enhance our understanding of this interesting biochemical process.

If indeed there are two transpeptidase activities in *S. aureus* (see earlier section), they may not both be needed for cell survival. This possibility is one interpretation which could be drawn from the report (180) that highly penicillin-resistant *S. aureus* mutants still bound penicillin at low concentrations. This binding would have to be to the more sensitive of the two putative transpeptidases, because binding was accomplished with penicillin at a concentration of 0.1 μg/ml. However, it has not yet been shown that the two *S. aureus* binding components correspond to two transpeptidase activities, nor has the sensitivity of the separated PBCs in the mutants been reported.

In *B. megaterium*, the transpeptidase activity assayed with diaminopimelic acid as acceptor may be essential to the viability of the organism but, if so, it is not the most sensitive vital target for the action of beta-lactam antibiotics. Cephalosporins such as 7-aminocephalosporanic acid and cephalothin killed cells at concentration at which little inhibition of the transpeptidation assayed with diaminopimelic acid as acceptor occurred (12% inhibition in the case of the 7-aminocephalosporanic acid) (240). The cross-linking activity, in contrast, showed better, although not excellent, agreement.

In *E. coli*, the cross-linking activity has the sensitivity to ampicillin, cephalothin, and methicillin expected for the killing site (217). Although the enzyme is 10-fold more sensitive to penicillin G than is the whole organism, this difference is attributed to the relative impermeability to this antibiotic of the outer membrane of *E. coli*. Although this explanation is probably correct, it should be noted that *E. coli* K-12 may be *more* permeable to penicillin G than ampicillin (196). The physiological role of the endopeptidase of *E. coli* has not been studied.

The study of transpeptidase activities in different organisms clarifies both their role in the cell and their relationship to the killing site. First, multiple transpeptidases seem to exist. Consequently, the assay of one, as is the case in either *E. coli* or *Streptomyces*, does not indicate without supporting evidence that it is either the only transpeptidase activity in that organism or that it is the killing site. If multiple transpeptidases are found, inhibition of merely the most sensitive enzyme may be lethal. Alternatively, inhibition of the most resistant may be necessary. This latter possibility would help explain results such as those found by Park et al. (180). Resistance of the second component would be obscured by the continued sensitivity of the first.

## Physiological Evidence for the Existence of Multiple Transpeptidases

The biochemical results agree with the physiological evidence that led to the initial hypothesis that multiple transpeptidases exist (212, 219, 225). In *E. coli*, low concentrations of penicillin are known to cause filamentation (59, 84, 85). Higher concentrations may inhibit elongation as well (212). With a penicillin-resistant mutant of *B. licheniformis*, conversely, elongation was inhibited by nonlethal concentrations of penicillin (99). In addition, structural differences apparently exist between cell walls from the ends and sides of the rod-shaped bacterium *B. subtilis* as evidenced by differential sensitivity to autolytic enzymes (73, 74). Moreover, intrinsic resistance to penicillin, unlike that to such drugs as streptomycin, is acquired only in a stepwise fashion (56, 57).

Separate transpeptidases could function for cell wall elongation, for septum formation, and for corner formation in rod-shaped organisms. This hypothesis would provide a rationale for the multiple PBCs (14, 15) and transpeptidase activities. Likewise, it would account for the inability to obtain mutants resistant in a single step to a high level of penicillin. If three transpeptidases had sensitivites of $x \leq y \leq z$,

then a single-step mutation would only yield mutants with resistance $\leq$ y. So far, such ideas remain speculation.

### Kinetics of Penicillin Inhibition

A variety of kinetic mechanisms for inhibition of different enzymes by penicillins have been described. In some cases penicillin acts as a reversible, competitive inhibitor. The soluble *E. coli* carboxypeptidase, which is inhibited in this manner, is extraordinarily sensitive to penicillins (107). Representative $K_I$ values are $1.6 \times 10^{-8}$ M for penicillin G, $6.4 \times 10^{-9}$ M for ampicillin, and $3 \times 10^{-6}$ M for cephalothin. Penicillins which either lacked the side chain, viz., 6-amino-penicillanic acid, or which had a hydrolyzed beta-lactam bond. viz., penicilloic acid, still retained some activity. The $K_I$ values of those compounds were $2.5 \times 10^{-6}$ M and $5 \times 10^{-6}$ M, respectively.

The second common type of inhibition is irreversible inactivation caused by covalent binding of the penicillin to the enzyme. This mode of action is exemplified by the *B. subtilis* carboxypeptidase (13, 233). Here, the reaction between penicillin and enzyme is believed to occur in a two-step process.

$$E + P \overset{K_I}{\rightleftharpoons} E\text{-}P \xrightarrow{k_3} EP^* \qquad (1)$$

The penicillin binds to the enzyme in a reversible complex; irreversible inactivation, probably by acylation of the enzyme, follows. For such a reaction

$$\ln(\text{active enzyme per total enzyme}) = -k_3 \cdot P \cdot t/(K_I + P). \qquad (2)$$

The values for $k_3$ and $K_I$ have been determined for 10 beta-lactam antibiotics (233). The sensitivity of the carboxypeptidase to inhibition appeared to depend primarily on its $K_I$ for the particular beta-lactam antibiotic. Values ranged from 28 to 0.1 mM. In contrast the rates of acylation, $k_3$, were relatively constant, $42\text{-}2 \times 10^{-2}$ s$^{-1}$. The actual numbers should be viewed with some caution, however. The values of $k_3$ correspond to one-half of the enzyme being converted from E–P to EP* in 1.7 to 35 s. Such times are of the same order as those required for mixing, degradation of free penicillin by penicillinase, etc. Indeed, the data plotted to determine $K_I$ and $k_3$ do not distinguish beyond doubt between the postulated mechanism and the simplistic model

$$E + P \xrightarrow{k_a} EP^* \qquad (3)$$

although the two-step mechanism is in all probability correct.

In any case, the concentrations of beta-lactam antibiotics customarily employed when measuring rates of binding are far below their $K_I$ values (reversible) for the enzyme. Consequently, the rate of inhibition can be approximated as

$$\ln(\text{active enzyme per total enzyme}) = -k_{app} \cdot P \cdot t \qquad (4)$$

where $k_{app} = (k_3/K_I)$. For the one-step mechanism (equation 3), an identical relation would hold, where $k_{app} = k_a$.

Briefly, equation 4 indicates that inactivation of the enzyme by acylation is proportional to time as well as to penicillin concentration, provided that the penicillin concentration is sufficiently greater than that of the enzyme, so that it remains constant over the course of the reaction. Consequently, when irreversible inactivation takes place, discussion of enzyme sensitivity merely in terms of penicillin concentration is relatively meaningless. Rates of inactivation are most easily determined by first binding penicillin to the enzyme for a fixed amount of time, then destroying free penicillin with penicillinase, and finally assaying residual enzymatic activity.

Although inhibition of carboxypeptidase in vitro is proportional to the exponential of the penicillin concentration, inhibition of the enzyme in growing cells is proportional to the concentration instead (13):

$$\text{active enzyme per total enzyme} = \frac{1}{1 + k_{app} \cdot P \cdot \text{generation time}}. \qquad (5)$$

The explanation is that in growing cultures the enzyme is being synthesized exponentially as well as inactivated exponentially. The combination of the two effects yields a nonexponential term dependent on the ratios of the relative rate constants.

The kinetics of irreversible inactivation have been demonstrated only with the carboxypeptidases from *B. subtilis* and *B. stearothermophilus* (13, 233, 245). Here, it was also confirmed that the inactivation of the enzyme was accompanied by the physical binding of penicillin (15, 232). Inhibition of a number of other enzymes, including the *E. coli* (106) and *M. luteus* (152) transpeptidases, is not reversed by penicillinase; but the kinetics of binding have not been studied, and there is no direct proof for the assumption that they are acylated.

The carboxypeptidases isolated from the penicillin-sensitive strains of *Streptomyces* R61 and K11 were inhibited by penicillin in a competitive manner (120, 123) (but see below).

However, that from strain R39 appeared to have been inhibited in a partially competitive fashion (123). The kinetics were mistakenly termed "noncompetitive" in the paper. As plotted, the data in fact show that both $V_{max}$ and $K_m$ were altered in the presence of penicillin. Moreover, additional data points would be desirable, because lines indicating strictly competitive inhibition might be drawn through the points illustrated. If in fact both $K_m$ and $V_{max}$ were altered, the binding of penicillin at the substrate site might distort the enzyme so as to result in a decrease in its catalytic efficiency ($V_{max}$). Such a phenomenon has been seen in the inhibition of penicillinases by some penicillins. Other possible problems are that approximately comparable distortions in kinetics would have been obtained if penicillin were causing irreversible inactivation of the enzyme (cf. 233), although the possibility of such inactivation was rendered fairly unlikely by extensive controls. Likewise, reversible inactivation would lead to similar distortions, provided that release of bound penicillin occurred slowly. Although the rate of release has not been reported for the R39 enzyme, for the R61 enzyme it is very slow, with a half-time of several hours (see below). Some hydrolysis of penicillin due to instability under the conditions of assay could have led to a distortion of the kinetics. Consequently, the nature of the inhibition of the R39 enzyme must be considered unresolved. However, confirmation that inhibition is partially competitive and an explanation of the phenomenon would be of major importance in shaping thinking about the nature of the interaction of penicillin with its receptors.

An additional type of inhibition which may occur is irreversible inhibition that is not accompanied by irreversible binding of penicillin. After inhibition of the D-alanine carboxypeptidase of *E. coli* by penicillin G, only 66% of the enzymatic activity was restored by addition of penicillinase (107). Although the authors suggest that penicilloic acid might account for the residual inhibition, this does not appear to be the case. The penicilloic acid concentration should only be 0.008 μg/ml; 50% inhibition of the enzyme by penicilloic acid only occurs at 2 μg/ml, a concentration 250-fold higher. Likewise, because the *E. coli* carboxypeptidase does not bind penicillins irreversibly (114), models involving partial binding or hydrolysis are unlikely. One alternative which has precedent in a related system is that the presence of penicillin may convert the enzyme to a particularly labile state, in which it then proceeds to denature. Such a phenomenon occurs when the *S. aureus* penicillinase is exposed to methicillin (35, 36, 96). Moreover, as has been shown recently, penicillin binding renders the *Streptomyces* R61 enzyme more readily denaturable (92).

Irreversible inhibition is not always irreversible (see earlier section). Release of penicillin G from the *B. subtilis* D-alanine carboxypeptidase at 37 C occurs with a half-time of 200 min (16). At 55 C, release from the *B. stearothermophilus* carboxypeptidase is even faster (half-time of 10 min). Because the incubation period for assay of enzymatic activity is often long compared with these release times, misleading kinetic results can be obtained. In the case of the *B. subtilis* carboxypeptidase, 6-aminopenicillanic acid caused partial inhibition at low concentrations. Very much higher concentrations did not inhibit the remaining activity (115). This result suggested the existence of two carboxypeptidases, one sensitive to 6-aminopenicillanic acid and the other resistant. In reality, there is only one enzyme. The "resistant fraction" was an artifact of release of 6-aminopenicillanic acid during the course of the assay.

Such artifacts do not account for the partial reversibility of the inhibition by penicillin of the transpeptidase activity assayed with diaminopimelic acid as acceptor in *B. megaterium*. Unlike the above example, the proportion of activity in the *B. megaterium* system which was reversible was independent of the length of time for which the penicillin-inhibited enzyme was exposed to penicillinase before assay (240). If hydrolysis were occurring, a greater proportion of the activity would appear to be reversible at later times.

In *B. subtilis*, the kinetics of penicillin binding to the PBCs I, IV, and V were those predicted for irreversible binding (15). However, the binding curve of component II was biphasic for certain penicillins, *viz.*, 6-aminopenicillanic acid and penicillin G. The reason for this behavior is not yet known. A trivial explanation is that there were actually two proteins in the component II peak obtained on sodium dodecyl sulfate polyacrylamide gels. However, this possibility seems unlikely in that the components would have needed identical molecular weights and rates of binding for a number of penicillins, and they would have had to be present in equal amounts. Other possibilities are either negative cooperativity in penicillin binding or else a difference in the accessibility to these penicillins unique to component II. In any case, the behavior of component II indicates that an electrophoretically homogene-

ous protein may bind penicillin at two separate rates. Consequently, a biphasic penicillin binding curve is not necessarily evidence for multiple proteins which bind penicillin or are inhibited by it.

Considerable care must be taken to exclude the possibility of acylation in "competitively" inhibited enzymes. Because covalent binding can be reversed, the actual reaction scheme is

$$E + P \xrightarrow{k_1} EP \xrightarrow{k_2} E + P^* \quad (6)$$

where $E$ = enzyme, $P$ = penicillin, $EP$ = penicilloylated enzyme, and $P^*$ is the unidentified produce of release. Provided that $P \gg E$ (so that the decrease in the concentration of $P$ as it is converted to $P^*$ can be neglected), then in the steady state

$$\frac{(E)(P)}{(EP)} = \frac{k_2}{k_1} = K_I^*. \quad (7)$$

An apparent $K_I$ can thus be determined. For the *B. subtilis* carboxypeptidase at 25 C (13, 16), the values for penicillin G are $k_2 = 1.1 \times 10^{-3}$/min, $k_1 = 4 \times 10^4$/M/min, $K_I^* = 2.7 \times 10^{-8}$ M: for 6-aminopenicillanic acid, $k_2 = 5.5 \times 10^{-3}$/min, $k_1 = 7.6 \times 10^2$/M/min, $K_I^* = 7.2 \times 10^{-6}$ M. These values of $K_I^*$ closely resemble the values of $K_I$ for reversibly inhibited carboxypeptidases. Moreover, although the *B. subtilis* (233) and *B. stearothermophilus* (16) carboxypeptidases are acylated, the kinetics of inhibition are competitive with respect to substrate. Thus, the existence of competitive kinetics in the absence of supporting data does not suffice to distinguish between mechanisms involving covalent and noncovalent inhibition.

The necessity for caution is emphasized by the behavior of the *Streptomyces* R61 carboxypeptidase. Penicillin binds to this enzyme slowly (accompanied by a conformational change in the enzyme). Penicillin is also released very slowly (half-time of 145 min) (92, 163). The reaction scheme is

$$E + P \underset{k_b}{\overset{k_f}{\rightleftharpoons}} EP \quad K_D = \frac{k_r}{k_f}. \quad (8)$$

At 25 C, in 10 mM sodium phosphate, pH 7.0, $k_f = 1.08 \times 10^6$/M/min, $k_r = 4.8 \times 10^{-3}$/min, $K_D = 4.5 \times 10^{-9}$ M. These values closely resemble those for an acylated carboxypeptidase, that from *B. subtilis*. Demonstration that acylation in fact is not occurring is thus imperative. Is penicillin released from the *Streptomyces* R61 carboxypeptidase as intact penicillin or as a rearrangement product, as takes place with the *B. stearothermophilus* enzyme? Is bound radioactive penicillin immediately released by sodium dodecyl sulfate?

## Models for the Interaction of Penicillin with Its Target

A number of models have been proposed which suggest an analogy between penicillin and various portions of the peptidoglycan. Before it was known that penicillin inhibited cross-linking, Collins and Richmond proposed that penicillin G was an analogue of *N*-acetylmuramic acid (43). The subsequent demonstration of the effect of penicillin on transpeptidation deprived this hypothesis of much of its attraction. Subsequently, Wise and Park argued that penicillin was an analogue of the L-alanyl-γ-D-glutamyl portion of acetylmuramylpentapeptide (243). However, this hypothesis was rendered unlikely by the finding that the α-carboxyl of the glutamate in the pentapeptide of *S. aureus* was amidated.

Tipper and Strominger (228) proposed that penicillin was an analogue of the terminal acyl-D-alanyl-D-alanine in the pentapeptide chain (see Fig. 7). More specifically, the fixed ring structure common to penicillins was hypothesized to be a structural analogue of that conformation of acyl-D-alanyl-D-alanine which binds to the active site of the enzyme. In this model, the reactive CO—N bond in the highly strained beta-lactam ring would correspond in position to the peptide bond cleaved during transpeptidation and would be ideally situated to react with the acyl acceptor site of the enzyme. The two rings of the penicillin nucleus are in different planes, and it was further hypothesized that penicillin might more strictly be an analogue of a transition state during cleavage of the normal peptide substrate, at which the planar double-bond character of the peptide bond had deteriorated into a nonplanar single-bonded state. Reaction of the transpeptidase with the beta-lactam ring of penicillin would result in formation of a penicilloyl enzyme complex analogous to the postulated acyl enzyme intermediate and would provide a rational explanation for the acylation of PBCs by beta-lactam antibiotics. The idea that penicillin was a transition state analogue was further explored by Lee (118).

Several lines of evidence support this model. (i) Substrate protects the D-alanine carboxypeptidase of *B. subtilis* against inactivation by penicillin (233). (ii) The enzyme is inhibited by sulfhydryl reagents (e.g., DTNB, mercurials), and either substrate or bound penicillin protects one sulfhydryl group on the native enzyme

against these reagents. (iii) The D-alanine carboxypeptidase of *B. stearothermophilus* appears to catalyze the transfer of bound penicillin to hydroxylamine (16). Such a reaction could best be explained if penicillin were bound at the catalytic site of the enzyme.

Although the most likely explanation is that both penicillin and substrate are bound at the catalytic site on the enzyme, containing an active sulfhydryl group, the data are also compatible with an alternative model in which the sulfhydryl is at an allosteric site. In this latter case, competition would be due to alternative conformational states of the enzyme (catalytically active and inactive) induced by substrate or penicillin. Experimentally, these possibilities can be distinguished by demonstrating that substrate and penicillin are bound at the same site (e.g., by affinity labeling or by isolation of the hypothetical acyl enzyme intermediate) or by crystallographic studies of the enzyme with substrate or inhibitor bound, both difficult objectives.

In addition, the substrate analogue hypothesis predicted that 6-methyl-penicillins and 7-methyl-cephalosporins, which would more closely resemble the D-alanyl-D-alanine, might be even better antibiotics than the unmodified molecule (228). This prediction has not been supported by experiment either in vivo (18, 102) or in vitro (240). However, the 7-methoxy substituents on cephalosporins result in highly active antibiotics which also are active in vitro as inhibitors of the penicillin-sensitive enzymes (102, 150, 151, 214). Thus, before the relationship of these data to the proposed mechanism can be evaluated, it is important to know why two bulky substituents on the beta-lactam ring (methyl and methoxy) have such different effects on activity. There are presently insufficient data relating to this point to evaluate it further. One important consideration may be the effect of different substituents on the reactivity of the beta-lactam ring.

The alternative hypothesis that penicillin may exert its control at an allosteric site on the enzyme is based on studies of the *Streptomyces* DD-carboxypeptidase-transpeptidases. Several experimental observations led to this hypothesis. (i) Inhibition of the R39 enzyme by penicillin is partially competitive rather than competitive (123). (ii) The binding of penicillin to the R61 enzyme, monitored fluorimetrically, can be competed with neither acceptor nor donor (92). (iii) Penicillin apparently binds to the R61 enzyme under conditions where the enzyme is partially denatured and inactive (viz., in 3.6 M guanidine-hydrochloride) (163). (iv) The albus G carboxypeptidase is insensitive to inhibition by penicillins, but it is inhibited by synthetic substrate analogues (122, 164).

None of the above arguments is persuasive. (i) The unusual kinetics reported for inhibition of the R39 enzyme may have several explanations, which have been discussed above. (ii) The claim that transpeptidation donor cannot compete for binding with penicillin to the R61 enzyme, determined by physical measurements (92, 163), is inconsistent with the kinetic measurements that donor and penicillin do compete (120). It is not clear from the published data whether the amount of substrate present ($5 \times K_m$) would have been sufficiently high to compete with inhibitor. (iii) The binding of penicillin to inactivated enzyme does not indicate that the substrate and drug-binding sites are different. Rather, it implies that sufficient damage to the enzyme has occurred so that certain catalytic functions are lost. Substrate may also bind to the partially denatured enzyme. (iv) The failure of penicillin to inhibit the *S. albus* G carboxypeptidase suggests only that this enzyme has a stricter binding site than the other enzymes. In fact it has the lowest $K_m$ for substrate of all of the *Streptomyces* carboxypeptidases. An analogous case may be dihydropteroate synthetase in sulfonamide-sensitive and -resistant bacteria. Sulfonamides are structural analogues of *p*-aminobenzoic acid, a substrate for this enzyme. In the resistant organisms, sulfonamides, the substrate analogues, bind to the enzyme much more poorly, but the binding of substrate itself is unaffected (172, 173). There are some obvious differences in some of the bond angles in substrate and penicillin. If penicillin is an analogue of the transition state of the terminal D-alanyl-D-alanine, and not of the ground state, it might fail to bind to a substrate binding site which required an unusually specific fit. Inhibition of this enzyme by synthetic substrate analogues which closely resemble substrate is compatible with this concept. It indicates that certain peptide analogues, such as substrate itself, can fit the binding site of this particular enzyme, something which the putative transition state analogue cannot do. On the other hand, penicillin binds to the penicillin-sensitive D-alanine carboxypeptidases of *E. coli* and *Streptomyces* strains R61, K11, and R39 much better than does substrate: the values of $K_i$ for penicillin G are $1.6 \times 10^{-8}$ M, $7.5 \times 10^{-8}$ M, $6 \times 10^{-8}$ M, and $1.4 \times 10^{-8}$ M, respectively. The values of $K_m$ are 0.6 mM (UDP-acetylmuramyl-pentapeptide as

substrate), 12 mM, 11 mM, and 0.8 mM (*N*,*N*-diacetyl-lysyl-D-alanyl-D-alanine as substrate), respectively. This result, that inhibitor binds much better than substrate, is characteristic of transition state analogues (124).

The behavior of the D-alanine carboxypeptidase from *B. stearothermophilus* membranes was interpreted to provide further support for the allosteric model of penicillin action (11). First, penicillin gave only partial inhibition of the membrane activity, 75 to 80%. This partial inhibition was reflected in hyperbolic plots of $K_m/V$. Secondly, certain treatments, e.g., storage at 4 or −20 C, led to apparent partial dissociation of penicillin sensitivity from enzyme activity. In general, the partial loss of enzymatic activity was accompanied by disproportionately greater loss of the ability of penicillin to inhibit the enzyme. For example, storage at −20 C for 4 weeks led to 61% loss of activity, but only 10% of this remaining activity could be inhibited by penicillin. Consequently, the absolute penicillin-resistant activity had increased from 20% of the original total to 34.6%. Similarly, after storage for 3 weeks at 0 C, the membrane activity had decreased to 43%. It remained 80% inhibitable by penicillin. However, if after storage the membranes were resuspended and sedimented by centrifugation, then the penicillin sensitivity was lost.

The authors interpreted these complicated results to indicate that penicillin bound to "at most only a portion of the catalytic site," or, alternatively, that it acted as an allosteric inhibitor. The possibility that there were two D-alanine carboxypeptidases, which differed in stability at 4 and −20 C and one of which was penicillin insensitive, was considered, although deemed unlikely. The authors argued that this model could not explain the increase in the total penicillin-resistant activity which occurred upon storage.

The behavior of the purified D-alanine carboxypeptidase of *B. stearothermophilus* (albeit from a different strain) provides no evidence for dissociation of activity and inhibition (245), and this purified enzyme is *totally* inhibitable by penicillin. Consequently, the presence of two carboxypeptidases and possible artifacts associated with the membrane location of the unpurified enzymes may account for the anomalous results reported (11). Several D-alanine carboxypeptidases are known to occur in both *E. coli* (224) and *B. megaterium* (240), and in the latter case one of these is penicillin insensitive.

If penicillin is a substrate analogue, then the active sites of carboxypeptidases can be divided into two classes according to whether acylation occurs or does not occur. (i) In the one studied example of the first class (*B. subtilis*), penicillin forms a weak reversible complex ($K_I = 10^{-2}$ to $10^{-4}$ M) with the enzyme. Significant inhibition results because of acylation. (ii) In the second class, penicillin binds very tightly ($K_I = 10^{-8}$ M), but does not acylate. The large difference in the values of $K_I$ in the two cases suggests one possible explanation for the occurrence or lack of acylation. In the first class, it is possible that the penicillin must be distorted to bind to the enzyme, hence the high $K_I$. This strain energy sufficiently activates the beta-lactam bond to induce acylation of an active group at the binding site of the enzyme, probably but not necessarily the same one involved in formation of the putative "acyl enzyme intermediate." In the second class, penicillin might fit the configuration of the active site much better. Because the molecule remains undistorted, acylation would not occur. Alternatively, of course, these two types of carboxypeptidases may have evolved with two different mechanisms, the second class having no acylation in either its mechanism or its inhibition. In other proteases, they are obviously examples of those which utilize covalent intermediates and those which do not (21).

Thus, there are at the present time two theories for the mode of inhibition of bacterial enzymes by penicillin—the substrate analogue hypothesis and the allosteric inhibitor hypothesis, neither of which is supported by substantial data. It has been shown that some bacterial enzymes at least are inhibited by an acylation mechanism and that some catalytic activities (transfer of pencilloyl to hydroxylamine and release of penicillin in an altered form) occur at this site, but it has not been decisively shown that this is the same site at which catalysis of substrate occurs. The protection by substrate against penicillin inactivation and the protection of a sulfhydryl group from inactivation by substrate are most simply interpreted by this model, but these facts are also compatible with other models. The possibility that penicillin may act at an allosteric site derives in part from the idea that there must be regulatory mechanisms in cell wall synthesis and that penicillin could be an analogue of some natural regulator (19). It seems likely that the major regulation of cell wall synthesis in most bacteria occurs at some earlier step in the pathway (such as a reaction involved in the synthesis of UDP-acetylmuramyl-pentapeptide), but that does not exclude the possibility that there may be

some modulation of cell wall synthesis at the terminal reactions, which are the reactions inhibited by penicillin. In fact there is now known a multiplicity of enzymes which are inhibited either reversibly or irreversibly. It would not be surprising to discover that the site of inhibition, i.e., the substrate site or an allosteric site, also was different in different cases.

## MECHANISM OF KILLING BY PENICILLINS

### Mutant Analysis

One way of better understanding the interaction of penicillin with its targets and the role of the enzymes involved in cell wall synthesis is by the examination of mutants. Two basic approaches have been attempted. In the first, penicillin-resistant mutants have been isolated. The findings in gram-positive organisms so far have been confusing and hard to interpret (see earlier section). With gram-negative organisms such as *E. coli*, penicillin resistance has generally been attributable either to alterations in penicillinase or permeability (19, 20, 29–31, 71, 72, 125, 126, 159, 166–170). Mutants in the penicillin-sensitive enzymes themselves have not been found.

The second approach has been the isolation of conditional lysis mutants (28, 34, 95, 130–132, 146, 156, 205, 241). The rationale here is that many of these should be temperature sensitive in the enzymes required for cell wall synthesis. The results have been generally disappointing. Almost all of the mutants isolated have been mutated in the enzymes involved in synthesis of the soluble precursors of the peptidoglycan. However, one mutant in *E. coli* appears to have a thermolabile particulate D-alanine carboxypeptidase (156). The soluble carboxypeptidase shows no change. A second mutant appears defective in an enzyme involved in the formation of lipid intermediate (156). More detailed characterization of the D-alanine carboxypeptidase mutant should prove illuminating. Several other mutants, which accumulate UDP-acetylmuramyl-pentapeptide and in which no enzymatic defect has been found so far, may also be of considerable interest.

A related, but more direct, approach for isolating mutants defective in the penicillin-sensitive enzymes of *E. coli* has recently been attempted (108). Because penicillin-resistant mutants were generally caused by alterations in permeability or penicillinase, whereas lysis mutants were generally altered in the wrong enzyme, a double-selection method was used. Mutants were isolated which were simultaneously penicillin-resistant at low temperature and lytic at high temperature. The reasoning was that a certain proportion of the mutations which rendered vital penicillin-sensitive enzymes resistant would simultaneously render the enzymes temperature labile. Most of the mutants which became resistant due to changes in permeability or penicillinase would presumably not lyse and could be discarded. By means of this technique, 500 mutants were isolated. Of these, a number possessed either hypo- or hyper-cross-linked peptidoglycan. Further characterization of these mutants is in progress.

### Evidence That Penicillin Kills by Inhibiting Cross-Linking

Penicillin is believed to kill bacteria by inhibiting peptidoglycan cross-linking. However, the only thorough studies that this is the mechanism of killing in vivo have been carried out in *S. aureus* (228, 229, 243). Here, good correlation was shown between killing by penicillin and inhibition of cross-linking. Likewise, penicillin inhibits cross-linking of *B. megaterium* cell wall in vivo (78). Whether this inhibition correlates well with killing has not yet been reported.

In the presence of penicillin, *Proteus mirabilis* is converted to spherical forms (L-forms) which are viable in the presence of sucrose. These L-forms are able to grow and multiply and initially can revert to bacilli upon removal of penicillin. The peptidoglycan composition of these penicillin-induced L-forms of *P. mirabilis* was similar in composition to that of the wild type (138, 139). However, whereas the L-form cell wall was completely broken down by lysozyme, that of the parent rod was only partially degraded. This result led H. H. Martin to suggest that "penicillin . . . prevented the formation of certain cross-linkages within the mucopolymer which are indispensable for the establishment of shape and mechanical stability." Later studies from the same laboratory cast doubt on this conclusion (109, 137). Incomplete digestion by lysozyme, rather than the presence of additional cross-linking, was found to account for the reduced breakdown. Moreover, in both parent and L-form, 32 to 34% of the diaminopimelic acid residues in the cell wall were susceptible to dinitrophenylation, i.e., there was no difference in cross-linking. If the effect of penicillin on *P. mirabilis* is due to an effect on cross-linking, it would have to be due to an effect on an extremely localized region of the wall, e.g., an effect on the enzymes responsi-

ble for the synthesis of the corners of the rod (cf. earlier section). Likewise, no change in the cross-linking of the cell walls of *E. coli* grown in the presence of penicillin was observed in the one case where this has been studied (212).

In view of the great differences which exist between bacteria both in their PBCs and in the enzymes which they possess, which are sensitive to penicillin, generalization of the results obtained in *S. aureus* is obviously not justified. In vitro and in vivo studies in the bacilli and in vitro studies in *E. coli* are consistent with the lethal action of penicillin being inhibition of cross-linking. However, it is possible in these cases that the inhibition of cross-linking is a subsidiary reaction which occurs in addition to the actual action of the antibiotic.

A still unexplained effect of penicillins on peptidoglycan synthesis in *B. megaterium* could be a clue to an additional mechanism (240). When in vitro peptidoglycan synthesis was inhibited by concentrations of cloxacillin just sufficient to prevent cross-linking, two unidentified lysozyme digestion products were formed which possessed mobilities greater than those of known disaccharide-peptides. The amounts produced were very large, up to 65% of the total peptidoglycan synthesized. At only fivefold-higher concentrations of cloxacillin, the amount of these products decreased dramatically to 20%. What relation these products bear to the killing action of cloxacillin is not known.

### Killing by Amidino Penicillin

Indeed, a new class of penicillins, amidino penicillins, have a mode of killing different from that of normal penicillins. The basic structure of this class of derivatives is shown in Fig. 15. The distinguishing feature is the replacement by an amidino linkage of the usual acylamino linkage attaching the side chain. The effect of the drug was striking in that gram-negative organisms such as *E. coli* were up to 100 times more sensitive than were such gram-positive organisms as *S. aureus* or *B. subtilis*. In the case of a typical penicillin derivative, ampicillin, the ratio is just the opposite. Furthermore, *E. coli* treated with low concentrations of amidino penicillin formed large spherical bodies which slowly lysed, regardless of whether sucrose was present or not. Ampicillin-treated cells, in contrast, form small spheroplasts which are protected by sucrose, but which lyse instantly if the osmotic support is removed (133, 148). At high concentrations of the amidino penicillin, *E. coli* is lysed in the same manner as by ampicillin.

It thus appears that amidino penicillin functions poorly as a penicillin which inhibits cross-linking. However, there must exist some other enzymatic activity in gram-negative organisms which is lacking in gram-positive organisms and which is exquisitely sensitive to the amidino penicillin. The effects on cell morphology make it likely, although not certain, that the inhibited activity is related to envelope synthesis. One possible candidate might be the enzyme which attaches the lipoprotein (22–27, 100, 101, 104, 141) to the peptidoglycan, presumably by a transpeptidation.

Recent work lends credence to this model (145, 178). *E. coli* transpeptidase, carboxypeptidase, and endopeptidase were not inhibited by lethal concentrations of amidino penicillin. High concentrations of amidino penicillin had no effect on the binding of [$^{14}$C]penicillin G to *E. coli* membranes. Mutants resistant to the amidino penicillin showed no cross-resistance to normal penicillin.

### Transfer of Penicillin Side Chain

Studies of the binding to gram-positive organisms of penicillins labeled in either the side chain ($^{14}$C) or nucleus ($^{35}$S) indicated that both portions of the molecule were bound to the PBCs (see earlier section). However, several papers suggest that the problem should be re-examined in gram-negative organisms. *E. coli* possesses an amidase which cleaves the side chain from the penicillin nucleus (5, 39–42, 192). The activity of this amidase may be considerable, up to fourfold greater than that catalyzed by the penicillinase of the organism. A correlation has been reported between the activity of penicillins against *E. coli* and the susceptibility of the penicillins to the amidase (110); the highly active penicillins were unusually good substrates. Under appropriate conditions, the *E. coli* amidase can synthesize amide bonds either directly or via a transpeptidation. Such an enzyme is therefore capable of transferring the penicillin side chain to amino acceptors in the cell. Such a reaction may actually occur.

The binding of [$^{14}$C]penicillin G to *P. mirabilis* was little inhibited by 6-aminopenicillanic acid (211). In contrast, a structural ana-

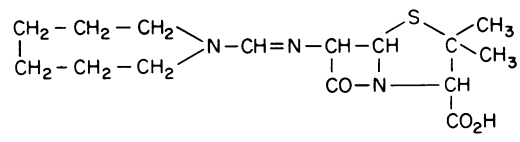


FIG. 15. *Structure of the amidino penicillin FL-1060, with which the studies described in the text were carried out.*

logue to the side chain, phenacetylglycine, prevented binding when present in only 10-fold excess. Moreover, although phenacetylglycine per se had no effect on cell growth, it prevented formation of spheroplasts in penicillin-treated cells. This result should be confirmed and extended.

## CONTROL OF CELL WALL SYNTHESIS

Synthesis of the peptidoglycan is coupled to that of the other macromolecules of the cell. It would therefore be expected to involve complicated regulatory pathways. Although this regulation is essentially not understood, the action of penicillin could be intimately intertwined with these regulatory mechanisms. As already discussed, penicillin causes the accumulation of UDP-acetylmuramyl-pentapeptide in some (but not all) organisms. An undefined mechanism must therefore exist by which the enzymes responsible for utilizing this nucleotide are modulated either by the degree of cross-linking which occurs or else directly by the penicillin.

In a phenomenon which is quite closely related, the amount of linear peptidoglycan which bacteria will synthesize is strongly affected by the action of penicillin. In such organisms as *S. aureus*, low concentrations of penicillin lead to synthesis of uncross-linked peptidoglycan. At higher concentrations, peptidoglycan synthesis virtually ceases (229). Such mechanisms are not universal. In *P. mirabilis*, peptidoglycan synthesis continues independent of the antibiotic concentrations (138). Such effects of penicillin on the net synthesis of peptidoglycan are reflected in in vitro assays. In the case of *E. coli*, methicillin and ampicillin inhibit total peptidoglycan synthesis in vitro; penicillin G does not (105). With *M. luteus*, penicillin G causes dramatic inhibition of synthesis (152). An unanswered question is whether penicillin might sometimes kill by inhibiting cell wall synthesis by such a control mechanism. If so, the anomalous results on the effect of penicillin on cross-linking in gram-negative organisms might be explained.

A third interesting example of control is the so-called zone phenomenon described by Eagle et al. (61, 62, 68). For some organisms, but not others, the rate at which penicillin kills the cells is decreased if the concentration of penicillin is increased beyond its optimal level. Two plausible explanations for this phenomenon exist. (i) Because synthesis of uncross-linked cell wall at low penicillin concentrations may be necessary for cell lysis, *S. aureus* H may be protected at high concentrations of penicillin because peptidoglycan synthesis is totally inhibited. (ii) Bacteria possess multiple PBCs, which vary in their affinities for penicillin. Inhibition of one component might lead to cell death, whereas inhibition of a less sensitive binding component, which might possess an antagonistic activity, would reduce the rate of killing. The *B. subtilis* system could provide an example of this second mechanism. At low penicillin concentration, the transpeptidase (presumably components I, II, and/or IV) is inhibited; higher penicillin concentrations inhibit the carboxypeptidase (component V) as well. If the action of the carboxypeptidase is indeed to limit the degree of peptidoglycan cross-linking, then its inhibition might well reduce the rate of bacteriolysis.

In some other organisms the antagonistic activity inhibited by penicillin at the higher concentration might be an autolysin such as the endopeptidase. Such an antagonistic action between autolysins and the cell wall synthetic enzymes has been clearly demonstrated in *Pneumococcus* (230). In this organism, the normal autolytic activity could be suppressed if cells were grown in the presence of ethanolamine rather than choline. Under such conditions, the rate at which penicillin killed cells was reduced 10-fold. Likewise, mutants of *B. subtilis* and *B. licheniformis* have been isolated which are highly deficient in autolytic activity (79). These mutants are lysed by two inhibitors of cell wall synthesis, vancomycin and cycloserine, only at concentrations 10- to 20-fold higher than those required to lyse the wild type (198).

An extremely interesting finding, which should be pursued vigorously, is the demonstration of mutant strains of *Staphylococcus* (10) and *Pediococcus cerevisiae* (237, 238, 242) which are penicillin dependent. Such organisms may have resulted from antagonistic, penicillin-sensitive activities becoming sufficiently out of balance during mutation to penicillin resistance so that partial inhibition of one activity became necessary for cell survival. Other models can of course be visualized, and determination of the actual explanation may have great importance for understanding the interaction of penicillin with the bacterial cell.

Induction of penicillinase synthesis may offer a promising and so far relatively unexploited approach to the study of regulation of cell wall synthesis. Irreversibly bound penicillin in *B. cereus* (6, 53, 58, 187, 188, 189, 191) induces penicillinase synthesis. Likewise, penicillinase is induced in *S. aureus* (103) in the presence of penicillin. Either the physical binding of the penicillin itself or perhaps an effect of penicillin

in inhibiting peptidoglycan synthesis leads to transfer of information to the cytoplasm and activation of penicillinase synthesis. Although the entire receptor-transmitter complex might be components of the penicillinase system, another possibility is that some genetic elements involved in control of cell wall synthesis may also be involved in regulation of penicillinase production (37, 38).

## SUMMARY

Great strides have been made in the past 30 years in understanding the mode of action of penicillin. Its possible targets have been defined. Methods have been developed for isolation of the different penicillin-sensitive activities. Several purified penicillin-sensitive enzymes are presently available in large amounts.

Future research will most likely focus on three issues. How does penicillin actually interact with its receptors? What are the in vivo functions of the different penicillin-sensitive enzymes? How does regulation of peptidoglycan synthesis occur, and what role does penicillin play in this process? Investigation of the mode of action is currently in a most exciting stage. Conflicting possibilities and hypotheses compel critical analysis. Technical knowledge has progressed to a level where these hypotheses can be tested adequately. The combined wealth of ideas and methods should promote rapid progress.

## LITERATURE CITED

1. Anwar, R. A., P. M. Blumberg, and J. L. Strominger. 1974. Penicillin binding components in *Bacillus subtilis* during sporulation. J. Bacteriol. **117**:924-925.
2. Araki, Y., A. Shimada, and E. Ito. 1966. Effect of penicillin on cell wall mucopeptide synthesis in an *Escherichia coli* particulate system. Biochem. Biophys. Res. Commun. **23**:518-525.
3. Araki, Y., R. Shirai, A. Shimada, N. Ishimoto, and E. Ito. 1966. Enzymatic synthesis of cell wall mucopeptide in a particulate preparation of *Escherichia coli*. Biochem. Biophys. Res. Commun. **23**:466-472.
4. Archibald, A. R., J. Baddiley, and N. L. Blumson. 1968. The teichoic acids. Advan. Enzymol. **30**:223-253.
5. Arcos, J. M., M. C. Ruiz, and J. D. Múgica. 1968. Beta-lactamase and penicillinacylase coexist in *Escherichia coli*. J. Bacteriol. **96**:1870-1872.
6. Aten, R. F., and R. A. Day. 1973. Penicillin-binding component of *Bacillus cereus*. J. Bacteriol. **114**:537-542.
7. Baddiley, J. 1968. Teichoic acids and the molecular structure of bacterial walls. Proc. Roy. Soc. Ser. B Biol. Sci. **170**:331-348.
8. Baddiley, J. 1970. Structure, biosynthesis, and function of teichoic acids. Accounts Chem. Res. **3**:98-105.
9. Baddiley, J. 1972. Teichoic acids in cell walls and membranes of bacteria, p. 35-77. *In* P. N. Campbell and F. Dickens (ed.), Essays in biochemistry, vol. 8. Academic Press, New York.
10. Barber, M. 1953. Penicillin-resistant and penicillin-dependent staphylococcal variants. J. Gen. Microbiol. **8**:111-115.
11. Barnett, H. J. 1973. D-alanine carboxypeptidases of *Bacillus stearothermophilus:* solubilization of particulate enzymes and mechanism of action of penicillin. Biochim. Biophys. Acta **304**:332-352.
12. Blumberg, P. M. 1974. Penicillin binding components of bacterial cells and their relationship to the mechanism of penicillin action. Ann. N.Y. Acad. Sci. **235**:310-325.
13. Blumberg, P. M., and J. L. Strominger. 1971. Inactivation of D-alanine carboxypeptidase by penicillins and cephalosporins is not lethal in *Bacillus subtilis*. Proc. Nat. Acad. Sci. U.S.A. **68**:2814-2817.
14. Blumberg, P. M., and J. L. Strominger. 1972. Isolation by covalent affinity chromatography of the penicillin binding components from membranes of *Bacillus subtilis*. Proc. Nat. Acad. Sci. U.S.A. **69**:3751-3755.
15. Blumberg, P. M., and J. L. Strominger. 1972. Five penicillin binding components occur in *Bacillus subtilis* membranes. J. Biol. Chem. **247**:8107-8113.
16. Blumberg, P. M., R. R. Yocum, E. Willoughby, and J. L. Strominger. 1974. Binding of [$^{14}$C]-penicillin G to the membrane-bound and the purified D-alanine carboxypeptidases from *Bacillus stearothermophilus* and *Bacillus subtilis* and its reversal. J. Biol. Chem. (in press).
17. Bogdanovsky, D., E. Bricas, and P. Dezélée. 1969. Sur l'identité de la mucoendopeptidase et de la carboxypeptidase I *d'Escherichia coli*, enzymes hydrolysant des liaisons de configuration D-D et inhibée par la penicilline. C. R. Acad. Sci. (Paris) Ser. D **269**:390-393.
18. Böhme, E. H. W., H. E. Applegate, B. Toeplitz, J. F. Dolfini, and J. Z. Gougoutas. 1971. 6-Methyl penicillins and 7-methyl cephalosporins. J. Amer. Chem. Soc. **93**:4324-4326.
19. Boman, H. G., K. G. Eriksson-Grennberg, J. Földes, and E. B. Lindström. 1967. The regulation and possible evolution of a penicillinase-like enzyme in *Escherichia coli*, p. 366-372. *In* V. V. Koningsberger and L. Bosch (ed.), Regulation of nucleic acid and protein biosynthesis. Amsterdam Elsevier Publ. Co., Amsterdam.
20. Boman, H. G., K. G. Eriksson-Grennberg, S. Normark, and E. Matsson. 1968. Resistance of *Escherichia coli* to penicillins. IV. Genetic study of mutants resistant to D,L-ampicillin

concentrations of 100 μg/ml. Genet. Res. **12**:169–185.
21. Boyer, P. D. (ed.) 1971. The enzymes, vol. III, Peptide bond hydrolysis (3rd ed.). Academic Press Inc., New York.
22. Braun, V., and V. Bosch. 1972. Repetitive sequences in the murein-lipoprotein of the cell wall of *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **69**:970–974.
23. Braun, V., and V. Bosch. 1972. Sequence of the murein-lipoprotein and the attachment site of the lipid. Eur. J. Biochem. **28**:51–69.
24. Braun, V., K. Hantke, H. Wolff, and G. Gerisch. 1972. Degradation of the murein-lipoprotein complex of *Escherichia coli* cell walls by *Dictyostelium* amoebae. Eur. J. Biochem. **27**:116–125.
25. Braun, V., and K. Rehn. 1969. Chemical characterization, spatial distribution, and function of a lipoprotein (murein-lipoprotein) of the *Escherichia coli* cell wall. The specific effect of trypsin on the membrane structure. Eur. J. Biochem. **10**:426–438.
26. Braun, V., and U. Sieglin. 1970. The covalent murein-lipoprotein structure of the *Escherichia coli* cell wall. The attachment site of the lipoprotein on the murein. Eur. J. Biochem. **13**:336–346.
27. Braun, V., and H. Wolff. 1970. The murein-lipoprotein linkage in the cell wall of *Escherichia coli*. Eur. J. Biochem. **14**:387–391.
28. Bukhari, A. I., and A. L. Taylor. 1971. Mutants of *Escherichia coli* with a growth requirement for either lysine or pyridoxine. J. Bacteriol. **105**:988–998.
29. Burman, L. G., and K. Nordström. 1971. Colicin tolerance induced by ampicillin or mutation to ampicillin resistance in a strain of *Escherichia coli* K-12. J. Bacteriol. **106**:1–13.
30. Burman, L. G., K. Nordström, and G. D. Bloom. 1972. Murein and the outer penetration barrier of *Escherichia coli* K-12, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. J. Bacteriol. **112**:1364–1374.
31. Burman, L. G., K. Nordström, and H. G. Boman. 1968. Resistance of *Escherichia coli* to penicillins. V. Physiological comparison of two isogenic strains, one with chromosomally and one with episomally mediated ampicillin resistance. J. Bacteriol. **96**:438–446.
32. Chapman, G. B., and J. Hillier. 1953. Electron microscopy of ultra-thin sections of bacteria. I. Cellular division in *Bacillus cereus*. J. Bacteriol. **66**:362–373.
33. Chatterjee, A. N., and J. T. Park. 1964. Biosynthesis of cell wall mucopeptide by a particulate fraction from *Staphylococcus aureus*. Proc. Nat. Acad. Sci. U.S.A. **51**:9–16.
34. Chatterjee, A. N., and F. E. Young. 1972. Regulation of the bacterial cell wall: isolation and characterization of peptidoglycan mutants of *Staphylococcus aureus*. J. Bacteriol. **111**:220–230.
35. Citri, N. 1971. Penicillinase and other beta-lactamases, p. 23–46. *In* P. D. Boyer (ed.), The enzymes, vol. 4, (3rd ed.). Academic Press Inc., New York.
36. Citri, N., and M. R. Pollock. 1966. The biochemistry and function of beta-lactamase (penicillinase). Advan. Enzymol. **28**:237–323.
37. Cohen, S., C. J. Gibson, and H. M. Sweeney. 1972. Phenotypic suppression of methicillin resistance in *Staphyloccus aureus* by mutant noninducible penicillinase plasmids. J. Bacteriol. **112**:682–689.
38. Cohen, S., and H. M. Sweeney. 1968. Constitutive penicillinase formation in *Staphylococcus aureus* owing to a mutation unlinked to the penicillinase plasmid. J. Bacteriol. **95**:1368–1374.
39. Cole, M. 1969. Hydrolysis of penicillins and related compounds by the cell-bound penicillin acylase of *Escherichia coli*. Biochem. J. **115**:733–739.
40. Cole, M. 1969. Deacylation of acylamino compounds other than penicillins by the cell-bound penicillin acylase of *Escherichia coli*. Biochem. J. **115**:741–745.
41. Cole, M. 1969. Penicillins and other acylamino compounds synthesized by the cell-bound penicillin acylase of *Escherichia coli*. Biochem. J. **115**:747–756.
42. Cole, M. 1969. Factors affecting the synthesis of ampicillin and hydroxypenicillins by the cell-bound penicillin acylase of *Escherichia coli*. Biochem. J. **115**:757–764.
43. Collins, J. F., and M. H. Richmond. 1962. A structural similarity between N-acetylmuramic acid and penicillin as a basis for antibiotic action. Nature (London) **195**:142–143.
44. Cooper, P. D. 1954. The site of action of penicillin. III. Effect of surface-active substances on penicillin uptake by *Staphylococcus aureus*. Biochim. Biophys. Acta **13**:433–438.
45. Cooper, P. D. 1954. The association of the penicillin binding component of *Staphylococcus aureus* with a lipid fraction. J. Gen. Microbiol. **10**:236–245.
46. Cooper, P. D. 1955. The site of action of penicillin: some properties of the penicillin-binding component of *Staphylococcus aureus*. J. Gen. Microbiol. **12**:100–106.
47. Cooper, P. D. 1955. The site of action of penicillin: some changes in *Staphylococcus aureus* during the first two hours growth in penicillin media. J. Gen. Microbiol. **13**:22–38.
48. Cooper, P. D. 1956. Site of action of radiopenicillin. Bacteriol. Rev. **20**:28–48.
49. Cooper, P. D., R. C. Clowes, and D. Rowley. 1954. A note on the use of radioactive penicillin. J. Gen. Microbiol. **10**:246–249.
50. Cooper, P. D., and D. Rowley. 1949. Investigations with radioactive penicillin. Nature (London) **163**:480–481.
51. Cooper, P. D., D. Rowley, and I. M. Dawson. 1949. Location of radioactive penicillin in *Staphylococcus aureus* after contact with the

drug. Nature (London) **164**:842–843.
52. Crane, L. J., G. E. Bettinger, and J. O. Lampen. 1973. Affinity chromatography purification of penicillinase of *Bacillus licheniformis* 749/c and its use to measure turnover of the cell bound enzyme. Biochem. Biophys. Res. Commun. **50**:220–227.
53. Csányi, V., G. Jacobi, and B. F. Straub. 1967. The regulation of penicillinase synthesis. Biochim. Biophys. Acta **145**:470–484.
54. Cuatrecasas, P., and C. B. Anfinsen. 1971. Affinity chromatography, p. 345–378. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 22. Academic Press Inc., New York.
55. Daniel, J. W., and M. J. Johnson. 1954. Properties of the penicillin binding component of *Micrococcus pyogenes*. J. Bacteriol. **67**:321–328.
56. Demerec, M. 1945. Production of staphylococcus strains resistant to various concentrations of penicillin. Proc. Nat. Acad. Sci. U.S.A. **31**:16–24.
57. Demerec, M. 1948. Origin of bacterial resistance to antibiotics. J. Bacteriol. **56**:63–74.
58. Duerksen, J. D. 1964. Localization of the site of fixation of the inducer, penicillin, in *Bacillus cereus*. Biochim. Biophys. Acta **87**:123–140.
59. Duguid, J. P. 1946. The sensitivity of bacteria to the action of penicillin. Edinburgh Med. J. **53**:401–412.
60. Dusart, J., A. Marquet, J.-M. Ghuysen, J. M. Frère, R. Moreno, M. Leyh-Bouille, K. Johnson, C. Lucchi, H. R. Perkins, and M. Nieto. 1973. DD-Carboxypeptidase-transpeptidase and killing site of β-lactam antibiotics in *Streptomyces* strains R39, R61, and K11. Antimicrob. Ag. Chemother. **3**:181–187.
61. Eagle, H. 1948. A paradoxical zone phenomenon in the bactericidal action of penicillin *in vitro*. Science **107**:44–45.
62. Eagle, H. 1951. Further observations on the zone phenomenon in the bactericidal action of penicillin. J. Bacteriol. **62**:663–668.
63. Eagle, H. 1953. The binding of penicillin in relation to its cytotoxic action, p. 1–9. Symposium on growth inhibition and chemotherapy, Rome, 6th International Congress on Microbiology.
64. Eagle, H. 1954. The binding of penicillin in relation to its cytotoxic action. I. Correlation between the penicillin sensitivity and combining activity of intact bacteria and cell-free extracts. J. Exp. Med. **99**:207–226.
65. Eagle, H. 1954. The binding of penicillin in relation to its cytotoxic action. II. The reactivity with penicillin of resistant variants of streptococci, pneumococci, and staphylococci. J. Exp. Med. **100**:103–115.
66. Eagle, H. 1954. The multiple mechanisms of penicillin resistance. J. Bacteriol. **68**:610–616.
67. Eagle, H., M. Levy, and R. Fleischman. 1955. The binding of penicillin in relation to its cytotoxic action. IV. The amounts bound by bacteria at ineffective, growth inhibitory, bactericidal, and maximally effective concentrations. J. Bacteriol. **69**:167–172.
68. Eagle, H., and A. D. Musselman. 1948. The rate of bactericidal action of penicillin *in vitro* as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. J. Exp. Med. **88**:99–131.
69. Edwards, J. R., and J. T. Park. 1969. Correlation between growth inhibition and the binding of various penicillins and cephalosporins to *Staphylococcus aureus*. J. Bacteriol. **99**:459–462.
70. Ellar, D. J., D. G. Lundgren, and R. A. Slepecky. 1967. Fine structure of *Bacillus megaterium* during synchronous growth. J. Bacteriol. **94**:1189–1205.
71. Eriksson-Grennberg, K. G. 1968. Resistance of *Escherichia coli* to penicillins. II. An improved mapping of the *amp A* gene. Genet. Res. **12**:147–156.
72. Eriksson-Grennberg, K. G., H. G. Boman, J. A. T. Jansson, and S. Thorén. 1965. Resistance of *Escherichia coli* to penicillins. I. Genetic study of some ampicillin-resistant mutants. J. Bacteriol. **90**:54–62.
73. Fan, D. P., and B. E. Beckman. 1973. Structural difference between walls from hemispherical caps and partial septa of *Bacillus subtilis*. J. Bacteriol. **114**:790–797.
74. Fan, D. P., M. C. Pelvit, and W. P. Cunningham. 1972. Structural difference between walls from ends and sides of the rod-shaped bacterium *Bacillus subtilis*. J. Bacteriol. **109**:1266–1272.
75. Few, A. V., P. D. Cooper, and D. Rowley. 1952. Reaction of penicillin with the staphylococcal cell wall. Nature (London) **169**:283–284.
76. Few, A. V., P. D. Cooper, and D. Rowley. 1953. Determination of radio-penicillin uptake by electrodialysis. Biochem. J. **53**:xix.
77. Fitz-James, P. 1960. Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. J. Biophys. Biochem. Cytol. **8**:507–528.
78. Fordham, W. D., and C. Gilvarg. 1974. Kinetics of cross-linking of peptidoglycan in *Bacillus megaterium*. J. Biol. Chem. **249**:2478–2482.
79. Forsberg, C., and H. J. Rogers. 1971. Autolytic enzymes in growth of bacteria. Nature (London) **229**:272–273.
80. Forsberg, C. W., and J. B. Ward. 1972. *N*-acetylmuramyl-L-alanine amidase of *Bacillus licheniformis* and its L-form. J. Bacteriol. **110**:878–888.
81. Frère, J. M., J. M. Ghuysen, H. R. Perkins, and M. Nieto. 1973. Molecular weight and amino acid composition of the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61. Biochem. J. **135**:463–468.
82. Frère, J. M., J. M. Ghuysen, H. R. Perkins, and M. Nieto. 1973. Kinetics of concomitant transfer and hydrolysis reactions catalyzed by

the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61. Biochem. J. **135**:483-492.
83. Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring. 1972. Inhibitors of bacterial cell wall synthesis, p. 49-120. *In* The molecular basis of antibiotic action. John Wiley & Sons, Inc., New York.
84. Gardner, A. D. 1940. Morphological effects of penicillin on bacteria. Nature (London) **146**:837-838.
85. Gardner, A. D. 1945. Microscopical effect of penicillin on spores and vegetative cells of bacilli. Lancet **ii**:658-659.
86. Ghuysen, J. M. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in metabolism. Bacteriol. Rev. **32**:425-464.
87. Ghuysen, J. M., L. Dierickx, J. Coyette, M. Leyh-Bouille, M. Guinand, and J. N. Campbell. 1969. An improved technique for the preparation of *Streptomyces* peptidases and N-acetylmuramyl-L-alanine amidase active on bacterial wall peptidoglycans. Biochemistry **8**:213-222.
88. Ghuysen, J.-M., M. Leyh-Bouille, R. Bonaly, M. Nieto, H. R. Perkins, K. H. Schleifer, and O. Kandler. 1970. Isolation of DD carboxypeptidase from *Streptomyces albus* G culture filtrates. Biochemistry **9**:2955-2961.
89. Ghuysen, J.-M., M. Leyh-Bouille, J. N. Campbell, R. Moreno, J.-M. Frère, C. Duez, M. Nieto, and H. R. Perkins. 1973. Structure of the wall peptidoglycan of *Streptomyces* R39 and the specificity profile of its exocellular DD-carboxypeptidase-transpeptidase for peptide acceptors. Biochemistry **12**:1243-1251.
90. Ghuysen, J.-M., M. Leyh-Bouille, J. M. Frère, J. Dusart, K. Johnson, A. Marquet, and R. Moreno. 1972. The *Streptomyces* DD-carboxypeptidase-transpeptidase system as a model for the study of penicillin action. 3rd International Symposium on Medicinal Chemistry, Milan, Italy.
91. Ghuysen, J.-M., M. Leyh-Bouille, J. M. Frère, J. Dusart, K. Johnson, M. Nakel, J. Coyette, H. R. Perkins, and M. Nieto. 1972. *Streptomyces* DD-carboxypeptidase-transpeptidases and mechanism of action of penicillin. *In* E. Muñoz, F. García-Ferrandiz, and D. Vazquez (ed.), Molecular mechanisms of antibiotic action on protein biosynthesis and membranes. Elsevier Scientific Publ. Co., New York.
92. Ghuysen, J.-M., M. Leyh-Bouille, J. M. Frère, J. Dusart, A. Marquet, H. R. Perkins, and M. Nieto. 1974. The penicillin receptor in *Streptomyces*. Ann. N.Y. Acad. Sci. **235**:236-268.
93. Ghuysen, J.-M., J. L. Strominger, and D. J. Tipper. 1968. Bacterial cell walls, p. 53-104. *In* M. Florkin and E. H. Stotz (ed.), Comprehensive biochemistry 26A. American Elsevier Publishing Co., New York.
94. Glaser, L. 1973. Bacterial cell surface polysaccharides. Annu. Rev. Biochem. **42**:92-112.
95. Good, C. M., and D. J. Tipper. 1972. Conditional mutants of *Staphylococcus aureus* defective in cell wall precursor synthesis. J. Bacteriol. **111**:231-241.
96. Gourevitch, A., T. A. Pursiano, and J. Lein. 1962. Inactivation of staphylococcal penicillinase by reaction with resistant penicillins. Nature (London) **195**:496-497.
97. Hahn, F. E., and J. Ciak. 1957. Penicillin-induced lysis of *Escherichia coli*. Science **125**:119-120.
98. Hartmann, R., J.-V. Höltje, and U. Schwarz. 1972. Targets of penicillin action in *Escherichia coli*. Nature (London) **235**:426-429.
99. Highton, P. J., and D. G. Hobbs. 1971. Penicillin and cell wall synthesis: a study of *Bacillus licheniformis* by electron microscopy. J. Bacteriol. **106**:646-658.
100. Hirashima, A., and M. Inouye. 1973. Specific biosynthesis of an envelope protein of *Escherichia coli*. Nature (London) **242**:405-407.
101. Hirashima, A., H. C. Wu, P. S. Venkateswaran, and M. Inouye. 1973. Two forms of a structural lipoprotein in the envelope of *Escherichia coli*. Further characterization of the free form. J. Biol. Chem. **248**:5654-5659.
102. Ho, P. P. K., R. D. Towner, J. M. Indelicato, W. A. Spitzer, and G. A. Koppel. 1972. Biochemical and microbiological studies on 6-substituted penicillins. J. Antibiot. **25**:627-628.
103. Imsande, J., J. W. Zyskind, and I. Mile. 1972. Regulation of staphylococcal penicillinase synthesis. J. Bacteriol. **109**:122-133.
104. Inouye, M., J. Shaw, and C. Shen. 1972. The assembly of a structural lipoprotein in the envelope of *Escherichia coli*. J. Biol. Chem. **247**:8154-8159.
105. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1966. Glycopeptide transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions. Proc. Nat. Acad. Sci. U.S.A. **55**:656-663.
106. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reaction in strains of *Escherichia coli*. J. Biol. Chem. **243**:3180-3192.
107. Izaki, K., and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIV. Purification and properties of two D-alanine carboxypeptidases from *Escherichia coli*. J. Biol. Chem. **243**:3193-3201.
108. Kamiryo, T., and J. L. Strominger. 1974. Penicillin-resistant temperature-sensitive mutants of *Escherichia coli* which synthesize hypo- or hyper-cross-linked peptidoglycan. J. Bacteriol. **117**:568-577.
109. Katz, W., and H. H. Martin. 1970. Peptide crosslinkage in cell wall murein of *Proteus mirabilis* and its penicillin-induced unstable L-form. Biochem. Biophys. Res. Commun. **39**:744-749.
110. Kaufmann, W. 1964. The possible implication of

a bacterial enzyme in the biochemical mode of action of penicillins on gram negative bacteria. Biochem. Biophys. Res. Commun. **14**:458-462.

111. Kayser, F. H., E. J. Benner, R. Troy, and P. D. Hoeprich. 1971. Mode of resistance against β-lactam antibiotics in stapylococci. Ann. N.Y. Acad. Sci. **182**:106-117.
112. Larsen, H. 1967. Biochemical aspects of extreme halophilism. Advan. Microbial Physiol. **1**:97-132.
113. Lawrence, P. J., M. Rogolsky, and V. T. Hanh. 1971. Binding of radioactive benzylpenicillin to sporulating *Bacillus* cultures: chemistry and fluctuations in specific binding capacity. J. Bacteriol. **108**:662-667.
114. Lawrence, P. J., and J. L. Strominger. 1970. Biosynthesis of the peptidoglycan of bacterial cell walls. XV. The binding of radioactive penicillin to the particulate enzyme preparation of *Bacillus subtilis* and its reversal with hydroxylamine or thiols. J. Biol. Chem. **245**:3653-3659.
115. Lawrence, P. J., and J. L. Strominger. 1970. Biosynthesis of the peptidoglycan of bacterial cell walls. XVI. The reversible fixation of radioactive penicillin G to the D-alanine carboxypeptidase of *Bacillus subtilis*. J. Biol. Chem. **245**:3660-3666.
116. Lederberg, J. 1956. Bacterial protoplasts induced by penicillin. Proc. Nat. Acad. Sci. U.S.A. **42**:574-577.
117. Lederberg, J., and J. St. Clair. 1958. Protoplasts and L-type growth of *Escherichia coli*. J. Bacteriol. **75**:143-160.
118. Lee, B. 1971. Conformation of penicillin as a transition state analog of the substrate of peptidoglycan transpeptidase. J. Mol. Biol. **61**:463-469.
119. Levine, B. B. 1965. Immunochemical mechanisms involved in penicillin hypersensitivity in experimental animals and in human beings. Fed. Proc. **24**:45-50.
120. Leyh-Bouille, M., J. Coyette, J. M. Ghuysen, J. Idczak, H. R. Perkins, and M. Nieto. 1971. Penicillin-sensitive DD-carboxypeptidase from *Streptomyces* strain R61. Biochemistry **10**:2163-2170.
121. Leyh-Bouille, M., J.-M. Ghuysen, R. Bonaly, M. Nieto, H. R. Perkins, K. H. Schleifer, and O. Kandler. 1970. Substrate requirements of the *Streptomyces albus* G DD-carboxypeptidase. Biochemistry **9**:2961-2970.
122. Leyh-Bouille, M., J.-M. Ghuysen, M. Nieto, H. R. Perkins, K. H. Schleifer, and O. Kandler. 1970. On the *Streptomyces albus* G DD-carboxypeptidase mechanism of action of penicillin, vancomycin, and ristocetin. Biochemistry **9**:2971-2975.
123. Leyh-Bouille, M., M. Nakel, J.-M. Frère, K. Johnson, J.-M. Ghuysen, M. Nieto, and H. R. Perkins. 1972. Penicillin sensitive DD-carboxypeptidases from *Streptomyces* strains R39 and K11. Biochemistry **11**:1290-1298.
124. Lienhard, G. E. 1973. Enzymatic catalysis and transition-state theory. Science **180**:149-154.
125. Lindqvist, R. C., and K. Nordström. 1970. Resistance of *Escherichia coli* to penicillins. VII. Purification and characterization of a penicillinase mediated by the R factor R1. J. Bacteriol. **101**:232-239.
126. Lindström, E. B., H. G. Boman, and B. B. Steele. 1970. Resistance of *Escherichia coli* to penicillins. VI. Purification and characterization of the chromosomally mediated penicillinase present in *amp A*-containing strains. J. Bacteriol. **101**:218-231.
127. Linnett, P. E., and J. L. Strominger. 1974. Amidation and cross-linking of the enzymatically synthesized peptidoglycan of *Bacillus stearothermophilus*. J. Biol. Chem. **249**:2489-2496.
128. Linnett, P. E., and J. L. Strominger. 1974. Biosynthesis and cross-linking of the γ-glutamyl glycine containing peptidoglycan of vegetative cells of *Sporosarcina ureae*. J. Biol. Chem. **249**:2497-2506.
129. Lüderitz, O. 1970. Recent results on the biochemistry of the cell wall lipopolysaccharides of *Salmonella* bacteria. Angew. Chem. **9**:649-663.
130. Lugtenberg, E. J. J., L. de Haas-Menger, and W. H. M. Ruyters. 1972. Murein synthesis and identification of cell wall precursors of temperature-sensitive lysis mutants of *Escherichia coli*. J. Bacteriol. **109**:326-335.
131. Lugtenberg, E. J. J., and A. van Schijndel-van Dam. 1972. Temperature-sensitive mutants of *Escherichia coli* K-12 with low activities of the L-alanine adding enzyme and the D-alanyl-D-alanine adding enzyme. J. Bacteriol. **110**:35-40.
132. Lugtenberg, E. J. J., and A. van Schijndel-van Dam. 1972. Temperature-sensitive mutants of *Escherichia coli* K-12 with low activity of the diaminopimelic acid adding enzyme. J. Bacteriol. **110**:41-46.
133. Lund, F., and L. Tybring. 1972. 6β-Amidinopenicillanic acids—a new group of antibiotics. Nature N. Biol. **236**:135-137.
134. Maass, E. A., and M. J. Johnson. 1949. Penicillin uptake by bacterial cells. J. Bacteriol. **57**:415-422.
135. Maass, E. A., and M J. Johnson. 1949. The relations between bound penicillin and growth in *Staphylococcus aureus*. J. Bacteriol. **58**:361-366.
136. Mäkelä, P. H., and B. A. D. Stocker. 1969. Genetics of polysaccharide biosynthesis. Annu. Rev. Genet. **3**:291-322.
137. Martin, H. H. 1963. Zusammensetzung der Stützmembran in der Zellwand von normalen Zellen und Penicillin-Sphäroplasten von *Protein mirabilis*. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. **191**:409-414.
138. Martin, H. H. 1964. Composition of the mucopolymer in cell walls of the unstable and stable L-form of *Proteus mirabilis*. J. Gen. Microbiol. **36**:441-450.
139. Martin, H. H. 1964. Chemical composition of

cell wall mucopolymers from penicillin-induced spheroplasts and normal cells of *Proteus mirabilis*. Proc. 6th Int. Congr. Biochem., New York (abstr.), p. 518.
140. Martin, H. H. 1966. Biochemistry of bacterial cell walls. Annu. Rev. Biochem. **35**:457-484.
141. Martin, H. H., H. D. Heilmann, and H. J. Preusser. 1972. State of the rigid-layer in cell walls of some gram-negative bacteria. Arch. Mikrobiol. **83**:332-346.
142. Matsuhashi, M., C. P. Dietrich, and J. L. Strominger. 1965. Incorporation of glycine into the cell wall glycopeptide in *Staphylococcus aureus:* role of sRNA and lipid intermediates. Proc. Nat. Acad. Sci. U.S.A. **54**:587-594.
143. Matsuhashi, M., C. P. Dietrich, and J. L. Strominger. 1967. Biosynthesis of the peptidoglycan of bacterial cell walls. III. The role of soluble ribonucleic acid and of lipid intermediates in glycine incorporation in *Staphylococcus aureus*. J. Biol. Chem. **242**:3191-3206.
144. Matsuhashi, M., M. Furuyama, and B. Maruo. 1969. Penicillin-sensitive D-alanine carboxypeptidase in *Anabaena variabilis*, a blue-green alga. Biochim. Biophys. Acta **184**:670-671.
145. Matsuhashi, S., T. Kamiryo, P. M. Blumberg, P. Linnett, E. Willoughby, and J. L. Strominger. 1974. Study of the mechanism of action and development of resistance to a new amidino penicillin. J. Bacteriol. **117**:578-587.
146. Matsuzawa, H., M. Matsuhashi, A. Oka, and Y. Sugino. 1969. Genetic and biochemical studies on cell wall peptidoglycan synthesis in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **36**:682-689.
147. Meadow, P. M., J. S. Anderson, and J. L. Strominger. 1964. Enzymatic polymerization of UDP-acetylmuramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala and UDP-acetylglucosamine by a particulate enzyme from *Staphylococcus aureus* and its inhibition by antibiotics. Biochem. Biophys. Res. Commun. **14**:382-387.
148. Melchior, N. H., J. Blom, L. Tybring, and A. Birch-Andersen. 1973. Light and electron microscopy of the early response of *Escherichia coli* to a 6β-amidinopenicillanic acid (FL 1060). Acta Pathol. Microbiol. Scand. Sec. B **81**:393-407.
149. Meynell, G. G., and E. Meynell. 1970. Theory and practice in experimental bacteriology, 2nd ed. p. 18. Cambridge University Press.
150. Miller, A. K., E. Celozzi, B. A. Pelak, E. O. Stapley, and D. Hendlin. 1972. Cephamycins, a new family of β-lactam antibiotics. III. In vitro studies. Antimicrob. Ag. Chemoth. **2**:281-286.
151. Miller, T. W., R. T. Goegelman, R. G. Weston, I. Putter, and F. J. Wolf. 1972. Cephamycins, a new family of β-lactam antibiotics. II. Isolation and chemical characterization. Antimicrob. Ag. Chemother. **2**:132-135.
152. Mirelman, D., R. Bracha, and N. Sharon. 1972. Role of the penicillin-sensitive transpeptidation reaction in attachment of newly synthesized peptidoglycan to cell walls of *Micrococcus luteus*. Proc. Nat. Acad. Sci. U.S.A. **69**:3355-3359.
153. Mirelman, D., R. Bracha, and N. Sharon. 1974. Studies on the elongation of bacterial cell wall peptidoglycan and its inhibition by penicillin. Ann. N.Y. Acad. Sci. **235**:326-347.
154. Mirelman, D., and N. Sharon. 1972. Biosynthesis of peptidoglycan by a cell wall preparation of *Staphylococcus aureus* and its inhibition by penicillin. Biochem. Biophys. Res. Commun. **46**:1909-1917.
155. Mitchell, P., and J. Moyle. 1951. Relationships between cell growth, surface properties and nucleic acid production in normal and penicillin-treated *Micrococcus pyogenes*. J. Gen. Microbiol. **5**:421-438.
156. Miyakawa, T., H. Matsuzawa, M. Matsuhashi, and Y. Sugino. 1972. Cell wall peptidoglycan mutants of *Escherichia coli* K-12: existence of two clusters of genes, mra and mrb, for cell wall peptidoglycan biosynthesis. J. Bacteriol. **112**:950-958.
157. Mohberg, J., and M. J. Johnson. 1958. Comparative study of the binding of synnematin and penicillin by bacterial cells. J. Bacteriol. **76**:385-392.
158. Mohr, V., and H. Larsen. 1963. On the structural transformations and lysis of *Halobacterium salinarium* in hypotonic and isotonic solutions. J. Gen. Microbiol. **31**:267-280.
159. Monner, D. A., S. Jonsson, and H. G. Boman. 1971. Ampicillin resistant mutants of *Escherichia coli* K-12 with lipopolysaccharide alterations affecting mating ability and susceptibility to sex-specific bacteriophages. J. Bacteriol. **107**:420-432.
160. Muñoz, E., and A. Marquet. 1972. Cooperative binding of penicillin G to cell membranes from *Streptomyces albus:* possible relationship with the mechanism of resistance to and the mechanism of action of the antibiotic. *In* E. Muñoz, F. Garcia-Ferrandiz, and D. Vazquez, (ed.), Molecular mechanisms of antibiotic action on protein biosynthesis and membranes. Elsevier, New York.
161. Muñoz, E., A. Marquet, V. Larraga, and J. Coyette. 1972. Isolation, partial characterization of the cytoplasmic membrane fraction of *Streptomyces albus* G and DD-carboxypeptidase localization. Arch. Mikrobiol. **81**:273-288.
162. Nayler, J. H. C. 1971. Structure-activity relationships in semi-synthetic penicillins. Proc. Roy. Soc. Ser. B **179**:357-367.
163. Nieto, M., H. R. Perkins, J.-M. Frère, and J.-M. Ghuysen. 1973. Fluorescence and circular dichroism studies on the *Streptomyces* R61 DD-carboxypeptidase-transpeptidase. Penicillin binding by the enzyme. Biochem. J. **135**:493-505.
164. Nieto, M., H. R. Perkins, M. Leyh-Bouille, J.-M. Frère, and J.-M. Ghuysen. 1973. Peptide inhibitors of *Streptomyces* DD-carboxy-

peptidases. Biochem. J. **131**:163–171.
165. Nikaido, H. 1968. Biosynthesis of lipopolysaccharide in gram-negative enteric bacteria. Advan. Enzymol. **31**:77–124.
166. Nördstrom, K., L. G. Burman, and K. G. Eriksson-Grennberg. 1970. Resistance of *Escherichia coli* to penicillins. VIII. Physiology of a class II ampicillin-resistant mutant. J. Bacteriol. **101**:659–668.
167. Nördstrom, K., K. G. Eriksson-Grennberg, and H. G. Boman. 1968. Resistance of *Escherichia coli* to penicillins. III. AmpB, a locus affecting episomally and chromosomally mediated resistance to ampicillin and chloramphenicol Genet. Res. **12**:157–168.
168. Normark, S. 1969. Mutation in *Escherichia coli* K-12 mediating spherelike envelopes and changed tolerance to ultraviolet irradiation and some antibiotics. J. Bacteriol. **98**:1274–1277.
169. Normark, S. 1970. Genetics of a chain-forming mutant of *Escherichia coli*. Transduction and dominance of the *envA* gene mediating increased penetration to some antibacterial agents. Genet. Res. **16**:63–78.
170. Normark, S., H. G. Boman, and E. Matsson. 1969. Mutant of *Escherichia coli* with anomalous cell division and ability to decrease episomally and chromasomally mediated resistance to ampicillin and several other antibiotics. J. Bacteriol. **97**:1334–1342.
171. O'Callaghan, C. H., S. M. Kirby, A. Morris, R. E. Waller, and R. E. Duncombe. 1972. Correlation between hydrolysis of the $\beta$-lactam bond of the cephalosporin nucleus and expulsion of the 3-substituent. J. Bacteriol. **110**:988–991.
172. Ortiz, P. J. 1970. Dihydrofolate and dihydropteroate synthesis by partially purified enzymes from wild-type and sulfonamide-resistant pneumococcus. Biochemistry **9**:355–361.
173. Ortiz, P. J., and R. D. Hotchkiss. 1966. The enzymatic synthesis of dihydrofolate and dihydropteroate in cell-free preparations from wild-type and sulfonamide-resistant pneumococcus. Biochemistry **5**:67–74.
174. Osborne, M. J. 1969. Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. **38**:501–538.
175. Park, J. T. 1952. Uridine-5'-pyrophosphate derivatives. I. Isolation from *Staphylococcus aureus*. J. Biol. Chem. **194**:877–884.
176. Park, J. T. 1952. Uridine-5'-pyrophosphate derivatives. II. A structure common to three derivatives. J. Biol. Chem. **194**:885–895.
177. Park, J. T. 1952. Uridine-5'-pyrophosphate derivatives. III. Amino acid-containing derivatives. J. Biol. Chem. **194**:897–904.
178. Park, J. T., and L. Burman. 1973. FL-1060: a new penicillin with a unique mode of action. Biochem. Biophys. Res. Commun. **51**:863–868.
179. Park, J. T., J. R. Edwards, and E. M. Wise. 1974. In vivo studies on the uptake and binding of beta-lactam antibiotics in relation to inhibition of wall synthesis and cell death. Ann. N.Y. Acad. Sci. **235**:300–309.
180. Park, J. T., M. E. Griffith, and I. Stevenson. 1971. Resistance to penicillin in mutants of a penicillinase-negative organism, *Staphylococcus aureus* H. J. Bacteriol. **108**:1154–1160.
181. Park, J. T., and M. J. Johnson. 1949. Accumulation of labile phosphate in *Staphylococcus aureus* grown in the presence of penicillin. J. Biol. Chem. **179**:585–592.
182. Park, J. T., and J. L. Strominger. 1957. Mode of action of penicillin. Biochemical basis for the mechanism of action of penicillin and for its selective toxicity. Science **125**:99–101.
183. Pelzer, H. 1963. Mucopeptidhydrolasen in *Escherichia coli*. B. I. Nachweis und Wirkungsspezifität. Z. Naturforsch. **18b**:950–956.
184. Perkins, H. R., M. Nieto, J.-M. Frère, M. Leyh-Bouille, and J.-M. Ghuysen. 1973. *Streptomyces* DD-carboxypeptidases as transpeptidases. The specificity for amino compounds acting as carboxyl acceptors. Biochem. J. **131**:707–718.
185. Perret, C. J. 1953. Note on the biosynthesis and isolation of $^{35}$S-labelled benzylpenicillin. J. Gen. Microbiol. **8**:195–197.
186. Pollock, J. J., J.-M. Ghuysen, R. Linden, M. R. J. Salton, H. R. Perkins, M. Nieto, M. Leyh-Bouille, J. M. Frère, and K. Johnson. 1972. Transpeptidase activity of *Streptomyces* D-alanyl-D carboxypeptidases. Proc. Nat. Acad. Sci. U.S.A. **69**:662–666.
187. Pollock, M. R. 1950. Penicillinase adaptation in *Bacillus cereus:* adaptive enzyme formation in the absence of free substrate. Brit. J. Exp. Pathol. **31**:739–753.
188. Pollock, M. R. 1953. Penicillinase adaptation and fixation of penicillin sulphur by *Bacillus cereus* spores. J. Gen. Microbiol. **8**:136–197.
189. Pollock, M. R. 1957. The activity and specificity of inducers of penicillinase production in *Bacillus cereus* strain NRRL 569. Biochem. J. **66**:419–428.
190. Pollock, M. R. 1971. The function and evolution of penicillinase. Proc. Roy. Soc. Ser. B **179**:385–401.
191. Pollock, M. R., and C. J. Perret. 1951. The relation between fixation of penicillin sulphur and penicillinase adaptation in *Bacillus cereus*. Brit J. Exp. Pathol. **32**:387–396.
192. Pruess, D. L., and M. J. Johnson. 1965. Enzymatic deacylation of $^{35}$S-benzylpenicillin. J. Bacteriol. **90**:380–383.
193. Reynolds, P. E. 1971. Peptidoglycan synthesis in bacilli. I. Effect of temperature on the *in vitro* system from *Bacillus megaterium* and *Bacillus stearothermophilus*. Biochim. Biophys. Acta **237**:239–254.
194. Reynolds, P. E. 1971. Peptidoglycan synthesis in bacilli. II. Characteristics of protoplast membrane preparations. Biochim. Biophys. Acta **237**:255–272.
195. Reynolds, P. E., and H. J. Barnett. 1974. Trans-

peptidases and DD-carboxypeptidases in bacilli. Ann. N.Y. Acad. Sci. (in press).
196. Richmond, M. H., and R. B. Sykes. 1973. The β-lactamases of gram-negative bacteria and their possible physiological role. Advan. Microbial Physiol. **9**:31–88.
197. Rogers, H. J. 1967. The inhibiton of mucopeptide synthesis by benzylpenicillin in relation to irreversible fixation of the antibiotic by *Staphylococci*. Biochem. J. **103**:90–102.
198. Rogers, H. J., and C. W. Forsberg. 1971. Role of autolysins in the killing of bacteria by some bactericidal antibiotics. J. Bacteriol. **108**: 1235–1243.
199. Rogers, H. J., and H. R. Perkins. 1968. Cell walls and membranes. Spon. London.
200. Rogolsky, M., P. J. Lawrence, and V. T. Hanh. 1973. Binding of radioactive benzylpenicillin to asporogenous mutants of *Bacillus subtilis* during postexponential growth. J. Bacteriol. **114**:220–227.
201. Rolinson, G. N. 1971. Bacterial resistance to penicillins and cephalosporins. Proc. Roy. Soc. Ser. B **179**:403–410.
202. Rowley, D., P. D. Cooper, P. W. Roberts, and E. L. Smith. 1950. The site of action of penicillin. I. Uptake of penicillin on bacteria. Biochem. J. **46**:157–161.
203. Rowley, D., J. Miller, S. Rowlands, and E. Lester-Smith. 1948. Studies with radioactive penicillin. Nature (London) **161**:1009–1010.
204. Russell, A. D., and R. H. Fountain. 1971. Aspects of the mechanism of action of some cephalosporins. J. Bacteriol. **106**:65–69.
205. Russell, R. R. B. 1972. Temperature-sensitive osmotic remedial mutants of *Escherichia coli*. J. Bacteriol. **112**:661–665.
206. Ryter, A., and O. E. Landman. 1964. Electron microscope study of the relationship between mesosome loss and the stable L state (or protoplast state) in *Bacillus subtilis*. J. Bacteriol. **88**:457–467.
207. Sabath, L. D., S. J. Wallace, and D. A. Gerstein. 1972. Suppression of intrinsic resistance to methicillins and other penicillins in *Staphylococcus aureus*. Antimicrob. Ag. Chemother. **2**:350–355.
208. Salton, M. R. J. 1964. The bacterial cell wall. Elsevier Publishing Co., New York.
209. Schepartz, S. A., and M. J. Johnson. 1956. The nature of the binding of penicillin by bacterial cells. J. Bacteriol. **71**:84–90.
210. Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**:407–477.
211. Schmid, R., and R. Plapp. 1972. Binding of $^{14}$C-penicillin G to *Proteus mirabilis*. Arch. Mikrobiol. **83**:246–260.
212. Schwarz, U., A. Asmus, and H. Frank. 1969. Autolytic enzymes and cell division of *Escherichia coli*. J. Mol. Biol. **41**:419–429.
213. Sharpe, A., P. M. Blumberg, and J. L. Strominger. 1974. D-Alanine carboxypeptidase and cell wall cross-linking in *Bacillus subtilis*. J. Bacteriol. **117**:926–927.
214. Stapley, E. O., M. Jackson, S. Hernandez, S. B. Zimmerman, S. A. Currie, S. Mochales, J. M. Mata, H. B. Woodruff, and D. Hendlin. 1972. Cephamycins, a new family of β-lactam antibiotics. I. Production by actinomycetes, including *Streptomyces lactamdurans* sp.n. Antimicrob. Ag. Chemother. **2**:122–131.
215. Storm, D. R., P. M. Blumberg, and J. L. Strominger. 1974. Inhibition of the *Bacillus subtilis* membrane-bound D-alanine carboxypeptidase by 6-aminopenicillanic acid covalently coupled to Sepharose. J. Bacteriol. **117**:783–785.
216. Strominger, J. L. 1959. The amino acid sequence in the uridine nucleotide-peptide from *Staphylococcus aureus*. C. R. Trav. Lab. Carlsberg **31**:181–192.
217. Strominger, J. L. 1970. Penicillin-sensitive enzymatic reactions in bacterial cell wall synthesis. Harvey Lect. **64**:179–213.
218. Strominger, J. L. 1973. The actions of penicillin and other antibiotics on bacterial cell wall synthesis. Johns Hopkins Med. J. **133**:63–81.
219. Strominger, J. L., P. M. Blumberg, H. Suginaka, J. Umbreit, and G. G. Wickus. 1971. How penicillin kills bacteria: progress and problems. Proc. Roy. Soc. Ser. B. **179**:369–383.
220. Strominger, J. L., K. Izaki, M. Matsuhashi, and D. J. Tipper. 1967. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions. Fed. Proc. **26**:9–22.
221. Strominger, J. L., J. T. Park, and R. E. Thompson. 1959. Composition of the cell wall of *Staphylococcus aureus:* its relation to the mechanism of action of penicillin. J. Biol. Chem. **234**:3263–3268.
222. Strominger, J. L., and D. J. Tipper. 1965. Bacterial cell wall synthesis and structure in relation to the mechanism of action of penicillins and other antibacterial agents. Amer. J. Med. **39**:708–721.
223. Strominger, J. L., and D. J. Tipper. Structure of bacterial cell walls: the lysozyme substrate. *In* E. F. Osserman, R. E. Canfield, and S. Beychak (ed.), Symposium on Lysozyme (in press).
224. Strominger, J. L., E. Willoughby, T. Kamiryo, P. M. Blumberg, and R. R. Yocum. 1974. Penicillin-sensitive enzymes and penicillin-binding components in bacterial cells. Ann. N.Y. Acad. Sci. **235**:210–224.
225. Suginaka, H., P. M. Blumberg, and J. L. Strominger. 1972. Multiple penicillin-binding components in *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli*. J. Biol. Chem. **247**:5279–5288.
226. Tipper, D. J. 1970. Structure and function of peptidoglycans. Int. J. Syst. Bacteriol. **20**:361–377.
227. Tipper, D. J. 1973. Bacterial cell walls, p. 121–205, 331–346. *In* G. D. Fasman and S. N.

Timasheff (ed.), Subunits in biological systems. Marcel Dekker Inc., New York.
228. Tipper, D. J., and J. L. Strominger. 1965. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc. Nat. Acad. Sci. U.S.A. **54**:1133-1141.
229. Tipper, D. J., and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XII. Inhibition of cross-linking by penicillins and cephalosporins: studies in *Staphylococcus aureus in vivo*. J. Biol. Chem. **243**:3169-3179.
230. Tomasz, A., A. Albino, and E. Zanati. 1970. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. Nature (London) **227**:138-140.
231. Umbreit, J. N., and J. L. Strominger. 1972. Solubilization, purification and characterization of a penicillin-sensitive D-alanyl-D-alanine carboxypeptidase from *Bacillus subtilis*. Fed. Proc. **31**:414 (abstr.).
232. Umbreit, J. N., and J. L. Strominger. 1973. D-alanine carboxypeptidase from *Bacillus subtilis* membranes. I. Purification and characterization. J. Biol. Chem. **248**:6759-6766.
233. Umbreit, J. N., and J. L. Strominger. 1973. D-alanine carboxypeptidase from *Bacillus subtilis* membranes. II. Interaction with penicillins and cephalosporins. J. Biol. Chem. **248**:6767-6771.
234. Umbreit, J. N., and J. L. Strominger. 1973. Relation of detergent HLB number to solubilization and stabilization of D-alanine carboxypeptidase from *Bacillus subtilis* membranes. Proc. Nat. Acad. Sci. U.S.A. **70**:2997-3001.
235. Warth, A. D., and J. L. Strominger. 1971. Structure of the peptidoglycan from vegetative cell walls of *Bacillus subtilis*. Biochemistry **10**:4349-4358.
236. Weidel, W., and H. Pelzer. 1964. Bagshaped macromolecules—a new outlook on bacterial cell walls. Advan. Enzymol. **26**:193-233.
237. White, P. J. 1968. A strain of *Pediococcus cerevisiae* which requires methicillin for growth. J. Gen. Microbiol. **50**:85-105.
238. White, P. J. 1968. A comparison of the cell walls of *Pediococcus cerevisiae* and of a substrain that requires methicillin for growth. J. Gen. Microbiol. **50**:107-120.
239. Wickus, G. G., and J. L. Strominger. 1972. Penicillin-sensitive transpeptidation during peptidoglycan biosynthesis in cell-free preparations from *Bacillus megaterium*. I. Incorporation of free diaminopimelic acid into peptidoglycan. J. Biol. Chem. **247**:5297-5306.
240. Wickus, G. G., and J. L. Strominger. 1972. Penicillin-sensitive transpeptidation during peptidoglycan biosynthesis in cell-free preparations from *Bacillus megaterium*. II. Effect of penicillins and cephalosporins on bacterial growth and *in vivo* transpeptidation. J. Biol. Chem. **247**:5307-5311.
241. Wijsman, H. J. W. 1972. A genetic map of several mutations affecting the mucopeptide layer of *Escherichia coli*. Genet. Res. **20**:65-74.
242. Wilkinson, B. J., and P. J. White. 1973. Wall synthesis by *Pediococcus cerevisiae* and by a substrain that requires methicillin for growth. J. Bacteriol. **116**:507-509.
243. Wise, E. M., and J. T. Park. 1965. Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. Proc. Nat. Acad. Sci. U.S.A. **54**:75-81.
244. Work, E. 1969. Biochemistry of bacterial cell walls. Lab. Pract. **18**:831-838.
245. Yocum, R. R., P. M. Blumberg, and J. L. Strominger. 1974. Purification and characterization of the thermophilic D-alanine carboxypeptidase from membranes of *Bacillus stearothermophilus*. J. Biol. Chem. (in press).